Session 6
Poster Presentations

Poster Organisers Dr R Murray
Dr J G White

DETECTION OF POTATO VIRUS Y USING THE LIGASE CHAIN REACTION (LCR), IN COMBINATION WITH A MICROTITRE PLATE BASED METHOD FOR PRODUCT DETECTION

K J O'DONNELL, E CANNING and L G A YOUNG
Scottish Agricultural Science Agency, East Craigs, Edinburgh, EH12 8NJ, UK
email: odonnell@sasa.gov.uk

ABSTRACT

The Scottish Agricultural Science Agency (SASA) carries out diagnostic tests for viruses on potato leaf and tuber samples in support of the Scottish Seed Potato Classification Scheme. Currently, routine detection of potato viruses in submitted leaf samples is carried out using ELISA (enzyme-linked immunosorbent assay). However, ELISA is not sufficiently sensitive to consistently detect the small amounts of virus present in primary infected tubers. In this case, the dormancy of the tubers must first be broken and then the resulting plants tested by ELISA, a process which can take several weeks.

In order to reduce this period we have evaluated nucleic acid amplification techniques which can theoretically achieve the level of sensitivity necessary for virus detection directly from tubers. In this paper we report on the development of an assay for PVY based on the ligase chain reaction (LCR), a method based on the ligase-mediated exponential amplification of DNA probes specific to target DNA (or cDNA) of the pathogen. We have combined the LCR assay with a microtitre plate based detection system which removes the need to run electrophoresis gels to detect products of the assay. This method has the possibility of combining the sensitivity and specificity of nucleic acid-based techniques with the automation of ELISA. Preliminary results are shown which compare the performance of LCR with the standard ELISA method.

INTRODUCTION

The Scottish Seed Potato Classification Scheme (SPCS) (Jeffries, 1986) specifies strict tolerances for the level of viruses such as potato virus Y (PVY) permitted in seed potatoes. The scheme is enforced by field inspection during the summer months, from which leaf samples from plants showing viral symptoms are sent to SASA for diagnosis. Virus titre from these samples is high enough for ELISA (Clark & Adams, 1977) to be the method of choice. ELISA based diagnostic

methods are simple to carry out, are easily automated, and are therefore suitable for large numbers of samples. However, they are not without drawbacks. Firstly, the specificity of ELISA is dependent upon the availability of suitable antibodies. There may be no antibody available to detect a specific strain - for example to distinguish between the C, O, N and NTN strains of potato virus Y. Secondly, the usefulness of ELISA is limited by its sensitivity. For example, in the case of the SPCS, in crops where high aphid levels have been detected late in the growing season, symptoms may not appear until the next year. Such crops are subject to post-harvest tuber testing. ELISA is not sufficiently sensitive to pick up the low levels of virus present in the tubers of such plants, therefore dormancy must be broken and ELISA carried out on the resulting leaflets, a process which takes 6-8 weeks.

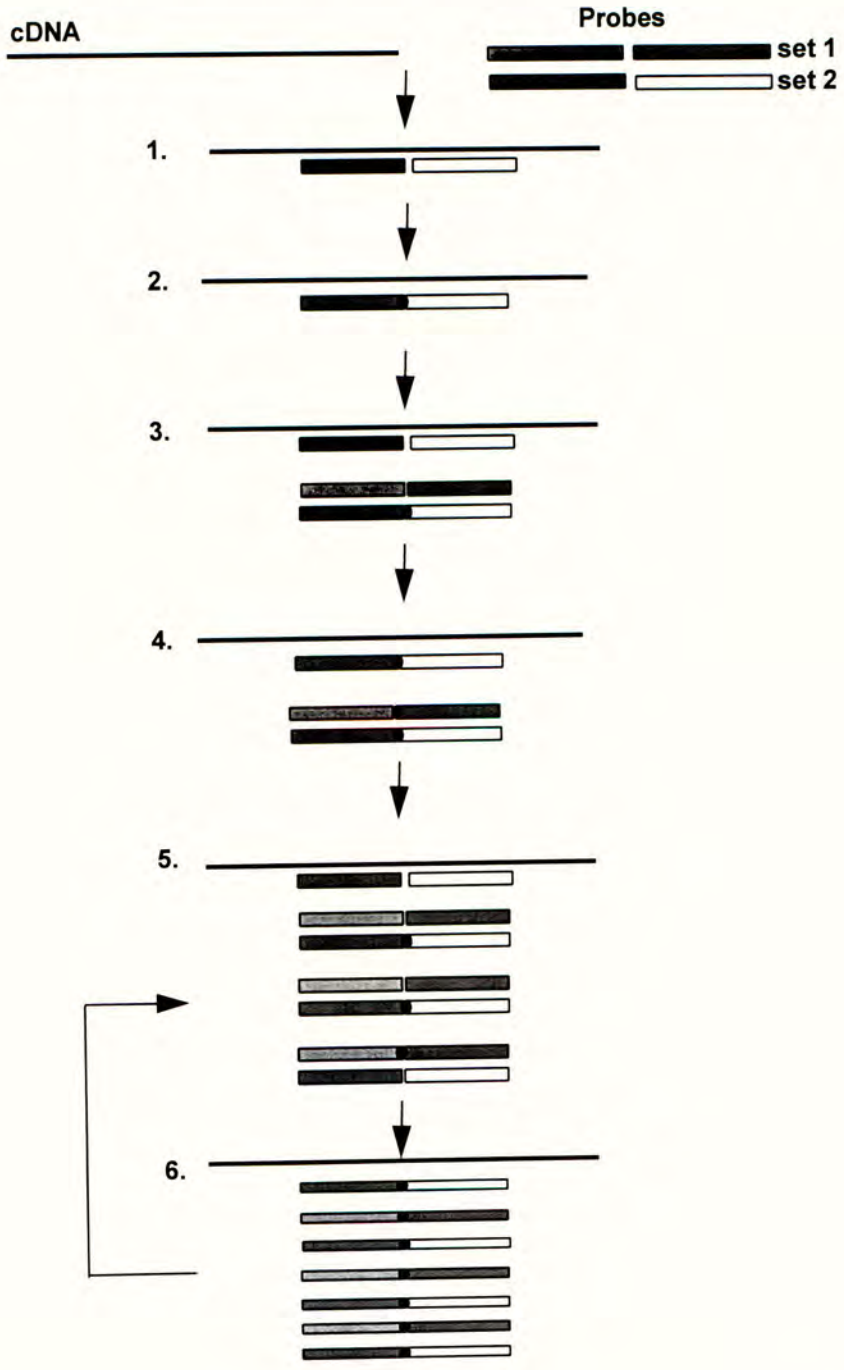
The need for increased specificity and sensitivity in assays for plant pathogens has led to interest in nucleic acid-based diagnostic methods such as the polymerase chain reaction (PCR) (Henson & French, 1993) As PCR is based on known nucleic acid sequences it can be very specific. In addition, the level of amplification achievable means that, in theory, it is capable of detecting single copies of pathogen genomes. One of the main obstacles to the introduction of PCR as a routine diagnostic method is that at present interpretation of the results depends on visualisation of the samples following agarose gel electrophoresis. This is a time-consuming and labour-intensive process which restricts the numbers of samples which can be handled.

The ligase chain reaction

A recently described method, LCR (Barany, 1991a, 1991b) offers the potential to combine the specificity and sensitivity associated with nucleic acid amplification with the ease of processing associated with ELISA. The LCR method is outlined in Figure 1. The method involves the use of two pairs of complementary probes. The two 25 nucleotide (nt) probes making up each pair are designed to anneal immediately adjacent to each other on one strand of the target DNA (or cDNA produced by reverse transcription of viral RNA) (1). The adjacent probes are ligated using a thermostable ligase to form a 50 nt product (2) which acts as a template in repeated cycles of denaturing, annealing and ligation (3-6). In the presence of target DNA the result is the exponential generation of the 50 nt ligation products which can be detected by visualisation on an agarose gel. Alternatively, the two oligonucleotide probes making up each pair can be labelled with different markers, opening up the possibility of detection by an easily automated ELISA-based method (Winn-Deen *et al.*, 1993).

LCR has been successfully used for the detection of human pathogens and point mutations leading to genetic disease (Wiedmann *et al.*, 1994) and has also been used for the detection of the plant pathogenic bacteria *Erwinia stewartii* (Wilson *et al.*, 1993). We report here the development of an LCR assay for the detection of PVY. The probes used have been labelled with biotin and digoxigenin, allowing

Figure 1. The ligase chain reaction



the development of a microtitre plate detection method which can be used in conjunction with standard automatic microtitre plate reading equipment.

MATERIALS AND METHODS

Reverse transcription

cDNA was generated from viral RNA using a reverse transcription protocol and kit from Pharmacia.

LCR reaction

LCR probes were designed to anneal to a 50 nucleotide conserved sequence of the PVY genome, generated by a alignment of PVY sequences retrieved from the EMBL and Genbank databases. The probe sequences were (5' to 3'):

M6819 biotinATGGCACATTTCGATGTTGCAG
M6820 PAAGCGTATATAGAAATGCGCAACAAdigoxigenin
M1963 PTCTGCAACATCTGAGAAATGTGCCATdigoxigenin
M1964 biotinTTGTTGCGCATTTCTATATACGCT

M6819 and M1963 were 5' phosphorylated. M6819/M6820 and M1963/M1964 formed two pairs of probes. When ligated, each made a 50nt stretch of DNA with a biotin molecule at one end and a digoxigenin at the other.

The LCR reaction conditions varied (see RESULTS) but always included 0.4 µl (20 U) thermostable ligase, 4 µl X 5 ligation buffer (both HT Biotechnology, Cambridge), LCR primers and probes and 1 µl of template (reverse transcription mix), in a total volume of 20 µl . The temperature cycling regime also varied as described in RESULTS and was carried out using a Perkin Elmer 9600 thermal cycler.

Microplate method

Microtitre plates were coated with streptavidin (Boehringer Mannheim)1/2000 in 50mM sodium carbonate buffer, for 1 hour at 37 °C. The plates were then washed twice with wash buffer A (WBA) (100mM Tris, 150 mM HCl, 0.05% Tween-20, pH 7.5). This was followed by the addition of a blocking buffer (1% BSA, 20mg/l herring sperm DNA in PBS) for 30 minutes at 37 °C. The plate was then washed twice with wash buffer B (WBB) (10 mM NaOH, 0.05% Tween-20). LCR reaction mix (20 µl) was added to 35 µl of sterile distilled water (SDW) and added to each well. The plate was then incubated for 30 minutes at 37 °C This was followed by 2 washes with WBA, 2 with WBB and a further 3 washes with WBA. Antidigoxigenin-alkaline phosphatase conjugate (50 µl) (Boehringer Mannheim) was added to each well, at 1/2000 in conjugate buffer (WBA plus 5%dried milk). The plate was incubated for 30 minutes at 37 °C and washed 4 times with WBA.

Substrate (50 µl)(1 mg/ml ρ-Nitrophenyl phosphate in 10% diethanolamine pH 9.8) was added to each well. After 1 hour, the plates were read on a Dynatech MR5000 plate reader, at 405 nm.

RESULTS

Development of the LCR assay

In optimising the LCR assay, particular attention was paid to the need to increase sensitivity but at the same time keeping background, non-specific ligation to a minimum. Variables which were altered included the cycling regime, the number of cycles, and the concentration of probes.

Longer ligation times, more cycles and a higher concentration of probe were all found to increase non-specific ligation. Higher temperatures and faster cycles were found to reduce it (results not shown). The optimal regime found was a reaction mix of: 0.4 µl (20 U) thermostable ligase, 4 µl X5 ligation buffer, 1 µl reverse transcription mix, 1 µl probes (2 pmol/µl) and 13.6 µl sterile distilled water. For negative controls, the RT mix was replaced by 1 µl SDW.

The optimal cycling regime was found to be: Initial incubations at 94 °C for 3 minutes and 60 °C for 3 minutes, followed by 27 cycles of 20 seconds at 92 °C (denaturation) 1 second at 60 °C (annealing) and 30 seconds at 65 °C (ligation).

Comparison of LCR and ELISA

An initial comparison of the microplate LCR method and standard ELISA was made using purified PVY. The results are shown in Table 1.

Table 1. Comparison of ELISA and LCR OD_{405} values

	virus concentration				
	100ng	10ng	1ng	100pg	-ve control
ELISA	2.02	0.95	0.20	0.08	0.09
LCR	0.46	0.20	0.15	0.10	0.04

The limit of ELISA is around 1 ng of purified PVY, whereas LCR can detect an order of magnitude lower.

CONCLUSIONS

We have developed a working LCR assay for potato virus Y, this being the first time the method has been used for the detection of a plant virus. These

preliminary results indicate that in terms of sensitivity, LCR showed at least a 10-fold improvement over standard ELISA techniques. Although we did not test the specificity of the method in these results, its use in the detection of point mutations shows that, when appropriate sequence data is available, its specificity is greater than that achieved with antibody-based methods. On the basis of these preliminary results, the microplate LCR assay for PVY has demonstrated increased sensitivity while retaining the possibility of a large throughput of samples using automated plate-reading equipment. Work is underway to assess both the performance of this method for detection of PVY in primary infected potato tuber samples and to further increase the sensitivity of the assay.

REFERENCES

Barany, F (1991a) Genetic disease detection and DNA amplification using a cloned thermostable ligase. *Proceedings of the National Academy of Science (USA).* **88**, 189-193.

Barany, F (1991b) The ligase chain reaction in a PCR world. *PCR Methods and Applications.* **1**, 5-16.

Clark, M F; Adams, A N (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology.* **34**, 475-483.

Henson, J M; French, R (1993) The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology.* pp.81-109.

Jeffries, C J (1986) The Scottish seed potato classification scheme and the production of nucleus stocks using micropropagation. In *BCPC Monograph No. 33, Healthy planting material: strategies and technologies,* D Rudd-Jones & F A Langton (eds), pp. 239-247.

Wiedmann, M; Wilson, W J; Czajka, J; Luo, J; Barany, F; Batt, C A (1994) Ligase chain reaction (LCR) - overview and applications. *PCR Methods and Applications* **3** S51-S64.

Wilson, W J; Wiedmann, M; Dillard, H R; Batt, C A (1993) Identification of *Erwinia stewartii* by a ligase chain reaction assay. *Applied and environmental Microbiology.* **60**, 278-284.

Winn-Deen, E S; Batt, C A; Wiedmann, M (1993) Non-radioactive detection of *Mycobacterium tuberculosis* LCR products in a microtitre plate format. *Molecular and Cellular Probes.* **7**, 179-186.

DETECTION AND IDENTIFICATION OF THE VIRUSES FORMING MIXED INFECTION IN GARLIC

R SALOMON, M KOCH, S LEVY and A GAL-ON
ARO Volcani Center, Bet-Dagan 50 250, Israel

ABSTRACT

Viral infection of many crops is often of mixed origin. In such infection, two or more viruses inhabit, propagate and spread in the plant organs. Such mixed infection may result in a synergystic effect, where the damage to the crop will be much greater than the damage caused by simply combining the damage caused by each individual virus. Damage to garlic yield by viral infestation is estimated as a reduction of 30-50% in weight and clove size. Garlic is only grown vegetatively and infestation with several different viruses has been reported. We have examined the Israeli cultivars "Shani" and "Shvat" for presence of both potyviruses and carla viruses. Extracts of garlic cloves from the above cultivars contain onion yellow dwarf potyvirus (OYDV), an additional potyvirus not yet completely classified, and at least two carlaviruses, probably garlic carlvirus-A and carlavirus-D. Some elephant garlic cultivars were found to contain the additional potyvirus, leek yellow stripe virus (LYSV), which was also found in some ornamental *Allium* species. Complementary DNA was synthesised from template RNA extracted from purified viruses isolated from leaves of the garlic cultivar "Shani". Three different clones harboring the coat protein gene were obtained. Two of these clones were identified as carrying segments of carlavirus coat gene. One clone is from a potyvirus, as was determined by sequence comparison. Controlled infection of virus-free garlic meristem cultures will reveal if a synergystic effect exists among the viruses infecting garlic, or whether the pathogenic agent OYDV causes most of the damage.

INTRODUCTION

Garlic (*Allium sativum*) is a common spice and the most abundant medicinal herb. Therefore it is an important crop worldwide. Since garlic is only propagated vegetatively, viruses infesting the crop are transmitted from one crop to the next via the propagation material. Viral infection reduces bulb and clove size. By comparing the yield of the same cultivar from traditionally propagated material and virus-free material from meristem culture, an increase in yield up to 50% was reported (Walkey, 1990). These findings emphasize the large economical advantage of virus-free garlic for propagation. Thus several countries are developing virus-free garlic by meristem culture and controlled propagation. In order to certify the propagation material, a

sensitive accurate and inexpensive detection method for all viruses infesting the crop is needed.

The most common sensitive and inexpensive procedures are serological, based on specific antibodies against all individual viruses infesting the plant under investigation. In Israel one cultivar, "Shani", comprises almost the entire garlic crop. Previously we have found that this cultivar is infected by onion yellow dwarf virus (OYDV) (Koch & Salomon, 1994). Recently we identified several additional viruses infecting the cultivar.

Several samples were infested with leek yellow stripe virus (LYSV). This virus is very common in elephant garlic (*Allium ampeloprasum*) and in several ornamental *Allium* species (including great headed garlic). In addition to these poyviruses, we did recover from great headed garlic (*A. ampeloprasum*) another potyvirus which was identified as turnip mosaic virus (TuMV) and is also very common in the cultivar "Shani". In addition to these potyviruses we were able to detect a carlavirus similar to garlic virus-1 (GV-1), Nagakubo *et al.* (1994), Choi *et al.* (1992). To purify LYSV and TuMV it was possible to propagate these viruses in specific test plants. However, OYDV exclusively infests garlic. Therefore we were unable to obtain a pure OYDV culture. In order to overcome biological difficulties in preparing a pure OYDV culture we designed several oligonucleotide primers, that were utilized to produce and multiply DNA complementary to the OYDV coat gene. The cDNA prepared was cloned and expressed in bacteria. The results of this work are described here.

MATERIALS AND METHODS

Viruses were extracted from leaves and cloves of garlic and the other *Allium* species studied. Purification was by polyethylene glycol (PEG) precipitation (Stein *et al.*,1986).

Mechanical inoculation was performed by rubbing viral extracts on carborundum predusted test plants of *Chenopodium amaranticolor* and produced local lesions by LYSV and TuMV inoculation respectively.

RNA was extracted as described before (Maniatis *et al.*, 1982; Gal-On *et al.*, 1990, 1992). Oligonucleotides were commercially produced by BTG Rehovot, Israel. DNA was synthesised by the cDNA synthesis kit of Boehringer-Mannheim. The cDNA was digested by phosphatase to form blunt ends and ligated into Bluescript plasmid. The PCR fragments were ligated to pUC57 plasmid utilizing the T-cloning kit (MBI fermentas) (Gal-On *et al.*, 1994; Salomon & Bernardi, 1995). Sequencing was performed by automated sequencer. Cloning and expression followed the described procedure (MEI Kit, pUC57 Plasmid). Antibody production and ACP-ELISA were done as previously described (Koch & Salomon, 1994).

RESULTS

All local cultivars of garlic tested were virus infested. Plant extracts tested with locally produced antiserum and this, together with antiserum against OYDV kindly provided by Dr. Herve Lot, indicated the presence of OYDV (Koch & Salomon, 1994). However, a potyvirus isolated from great headed garlic clones of *A. ampeloprasum,* did not react with OYDV antiserum. The antiserum against this second virus, later identified as TuMV, responded positively with many samples from the common garlic cultivar "Shani". Therefore the results point to the presence of more than one potyvirus in locally grown garlic.

From elephant garlic clones of *A. ampeloprasum* and from several ornamental *Allium* species we isolated an additional potyvirus identified by specific antiserum as LYSV (Gera *et al.*, personal communication). We thus found that some of our garlic crop is infected by three different potyviruses.

OYDV was found to infect only garlic, and it is therefore impossible to isolate it from the virus mixture by inoculation, to a specific test plant (van Dijk, 1993). To overcome this biological barrier we employed a molecular approach. cDNA against viral RNA extracted from the cultivar "Shani" was prepared, and the longest band isolated from the DNA mixture was cloned. Upon sequencing the cloned product, a similarity was found to the carlaviruses GV-A and GV-D by the "Gene Bank" comparison (Nagakubo *et al.*, 1994). Since the use of cDNA did not enable us to isolate any of the potyviruses from the mixture, a PCR procedure was utilized for this purpose. Primers complementary to conserved regions in the potyvirus coat protein (CP) gene and NIb gene were prepared. Utilizing these primers for PCR enabled us to obtain DNA segments from the cDNA encompassing part of the NIb gene and part of the CP gene. The whole CP gene alone was obtained when specific coat gene primers were utilized according to the sequence described by Nagakubo *et al.* (1994).

The similarity between the sequence of the cloned NIb-CP from garlic viral RNA and the previously reported GV-2 sequence of a potyvirus CP gene from Japanese garlic, was greater than 95% (Nagakubo *et al.*, 1994). The sequence of the 289 amino acids residues of the CP are summarized in Figure 1. Although the overall sequence similarity is >95%, most changes were found in the variable N-terminal region of the CP (8 out of 49 amino acids), thus indicating the existence of a different strain in Israel (Fig.1).

DISCUSSION

The analysis of the viruses infecting garlic in Israel, especially potyviruses, revealed the existence of three different potyviruses: OYDV, TuMV and LYSV. In addition we identified two carlaviruses identical to GV-A, and GV-D, in the virus mixture ("Gene Bank", Nagakubo *et al.,* 1994). The major difficulty encountered in this research was the separation of OYDV from the other viruses. The OYDV of garlic is exclusively

Figure, 1. The CP amino acid sequence of an Israeli isolated garlic potyvirus.

```
ANNELDAGMQTSKKQKDSTDKSIEQRDPASSQVNS
   D                        GN          S
QG KRDGEGSSGLSRNKDRDVNVGTTGTFSVPRIKQ
L   K       G   M
ISQKGISIPMDGGKSILNLDHLLQYKPNQLNISNTRA
 P
TVAQFKTWMERVQEDYGVTKSEMGIILNGLMVWCI
                                 G
ENGTSPNINGTWTMMDGDEQVAYPLRPIVEHAKPT

LRQ IMAHFSALAEAYIEMRNSEQAYMPRYGLQRNLT

DMGLARYAFDFYEVTSRTPVRAREAHAQMKAAALR

NSMPRLFGLDGNVTTTDEDTERHTAHDVNARMHHL
   R                          V
DGRHMQ
```

The amino acids in the lower lane represent the different amino acids in the Japanese isolate (GV-2). The underlined amino acids represent the aphid recognition domain, the begining of the core and the c-terminal region.

infecting garlic, and therefore it was impossible to isolate it from the other viruses in the mixture by infecting a specific test plant (van Dijk, 1993). Infecting virus-free garlic or another susceptible *Allium* species will not act as a biological filter for the isolation of OYDV. Thus we had to resort to a molecular approach utilizing specific oligonucleotide primers for PCR procedure. The DNA products were cloned, propagated and separated by gel electrophoresis, then sequenced by automated sequencing procedure.

Sequence comparison enabled us to identify the origin of the viral DNA. The sequence obtained was different from the already known sequence of TuMV. Sequence comparison of the PCR-DNA product showed a high level of similarity to GV-2 (Nagakubo *et al.*, 1994). However since 8 out of 12 differing amono acids were located at the variable N-terminal region of the CP, we assumed that the Israeli isolate is just a different strain of the same virus species. The conserved core region of the CP had only two amino acids changed out of 221.

Nagakubo *et al.*, (1994) indicate that GV-2 may be a Japanese garlic strain of LYSV. However there is no information to rule out the possibility that this virus is OYDV. To find out which of these two potyviruses is similar to GV-2, the cloned DNA was subcloned into an expression vector and expressed in bacteria. Purified bacterial CP will be utilized to produce antibodies, which will be compared to the existing antibodies against LYSV purified from test plants, and against OYDV from garlic, in a mixed population of viruses. Utilising molecular methods will enable us to develop specific antibodies against each of the potyviruses in the mixture of viruses affecting garlic. Additionally this sensitive method (RT-PCR), will help in the production of meristem cultured virus-free garlic.

ACKNOWLEDGMENTS

Contribution from the Agricultural Research Organisation, Volcan Center, Bet-Dagan 50 250, Israel, No 1770-E, 1995 series.

REFERENCES

Choi J N; Ueom T S; Choi Y D; Lee J S (1992) Molecular cloning of cDNA for garlic mosaic genom. *Korean Journal of Botany.* **35**, 253-257.

Gal-On A; Antignus Y; Rosner A; Raccah B (1991) Infectious *in vitro* RNA transcripts derived from cloned cDNA of the cucurbit potyvirus, Zucchini yellow mosaic. *Journal of General Virology.* **72**, 2639-2643.

Koch M; Salomon R (1994) Serological detection of onion yellow dwarf virus in garlic. *Plant Disease.* **78**, 785-788.

Maniatis T; Fritsch H F; Sambrook J (1982) *Molecular cloning: A laboratory manual* **1**. 3.35-3.51. New York: Cold Spring Harbor Laboratory.

Nagakubo T; Kubo M; Oeda K (1994) Nucleotide sequences of the 3' regions of two major viruses from mosaic-diseased garlic:Molecular evidence of mixed infection of potyvirus and carlavirus. *Phytopathology.* **86**, 640-645.

Salomon R; Bernardi F (1995) Inhibition of viral aphid transmission by N-terminus of the maize dwarf mosaic virus coat protein. *Virology.* **213**, 676-679.

Stein A; Salomon R; Cohen J; Loebenstein G (1986) Detection and characterisation of bean yellow mosaic virus in corms of *Gladiolus grandiflorus. Annals of Applied Biology.* **109**, 147-154.

van-Dijk P (1993) Survey and characterization of potyviruses and their strains of *Allium* species. *Netherlands Journal of Plant Pathology Supplement* **2**, 1-48.

Walkey DGA (1990) Virus diseases. In: *Onions and allied crops* pp. 191-212. H D Rabinowitch and J L Brewster (eds.) CRC press Boca Raton, Fl.

COMPARISON OF NOVEL MOLECULAR METHODS FOR THE DETECTION OF BEET NECROTIC YELLOW VEIN VIRUS (BNYVV)

C M HENRY, I BARKER, J MORRIS & S A HUGO
Central Science Laboratory, MAFF, Hatching Green, Harpenden, Herts, AL5 2BD, UK.

ABSTRACT

Four nucleic acid amplification techniques, RT-PCR, immunocapture RT-PCR, self-sustained sequence replication (3SR) and RT-PCR using nested primers, were compared with two TAS-ELISA techniques for sensitivity of detection of beet necrotic yellow vein virus (BNYVV). The most sensitive method for the detection of BNYVV in *C. quinoa* or sugar beet roots proved to be RT-PCR using nested primers followed by, in descending order of sensitivity, RT-PCR, immunocapture RT-PCR, amplified ELISA, TAS-ELISA and 3SR. The most sensitive technique, RT-PCR using nested primers, produced a specific PCR product of 326 base pairs in size but also an additional 450 base pair product. This technique gave an increase in sensitivity of 1000 times when compared with RT-PCR using one set of primers. Further work needs to be done to optimise this technique for the detection of BNYVV in sugar beet roots from soil.

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV) is the causal agent of Rhizomania disease of sugar beet (Tamada & Baba,1973). Detection of this virus in sugar beet and soil is an integral part of strategies for controlling the spread of the disease in England. Whilst detection of the virus in plants can be done efficiently using serological methods (ELISA), its detection in soil has been less easy. Detection in soil is normally based on a seedling bait test method where seedlings are grown in soil for a period of time and their roots are then tested for viral infection using ELISA. A diagnostic test based on reverse transcription followed by polymerase chain reaction (RT-PCR) has been developed (Henry *et al*, 1995). A specific 500 base pair fragment was amplified from the read-through region of the coat protein gene located on RNA 2. This method gave a sensitivity of 800 times that of a TAS-ELISA method and 50 times that of an amplified TAS-ELISA method. Use of this method in routine diagnostic tests enabled a reduction in the time needed for detection of Rhizomania in soil from 7 to 4 weeks.

Recently, other nucleic amplification techniques have been used for virus detection. The use of antibodies in immunocapture RT-PCR to selectively trap virus particles (Wetzel *et al*, 1991) may offer a new approach, overcoming problems encountered previously with RNA extraction and the removal of inhibitors of the PCR reaction. Nested primer technology; the use of a second round of PCR using a second set of primers, internal to the first pair, may offer advantages of increased sensitivity (Liu *et al*, 1991). Self-sustained sequence replication or 3SR is an isothermal transcription-based amplification technique for RNA (Fahy *et al*, 1991) which does not require the use of a thermal cycler. This paper reports progress on the use of the above techniques for the detection of BNYVV.

MATERIALS AND METHODS

Virus isolates used

Virus-infected *Chenopodium quinoa* leaves were produced by mechanical inoculation of BNYVV to plants using infected sap and Celite. A French isolate (Fr) of BNYVV was used (supplied by C Putz of INRA Colmar). Infected sugar beet roots contained a UK BNYVV isolate originating from West Stow, Suffolk.

Nucleic acid extraction, reverse transcription and PCR (RT-PCR)

The method followed was that of Henry *et al* (1995). An additional clean-up step to remove inhibitory substances from sugar beet root extracts was carried out using either a Qiagen 'RNeasy' kit or National Scientific Supply Co Inc USA's 'Crystal Clear' kit. *C. quinoa* nucleic acid extracts were prepared using either the method of Henry *et al* (1995) or that of Mumford (1994). RT-PCR reactions were carried out using two specific 20 base pair primers complementary to nucleotides 1781-1800 and homologous to nucleotides 1301-1320 of the RNA2 sequence published by Bouzoubaa *et al* (1986).

PCR using nested primers

One microlitre of nucleic acid extract was added to the reverse transcription mix used for RT -PCR. Initial nested primer evaluation was carried out using the RT-PCR method for 25 cycles. One microlitre of the RT-PCR product was added to a nested primer reaction mix. The amplification was carried out using an adaptation of the methods of Liu *et al* (1991) and a Perkin-Elmer kit. Three 20 base pair primers were synthesised for evaluation. These were complementary to nucleotides 1673-1692 (primer 21516) and 1681-1700 (primer 21517) and homologous to nucleotides 1375-1394 (primer 21515) of RNA 2 respectively. The sequences of the primers were as follows: Upstream primer; 21515; 5' ATA-GAG-CTG-TTA-GAG-TCA-CC 3'; Downstream primer; 21516; 5' AGC-AGC-CAT-AGC-AAC-AGC-TG 3'; Downstream primer; 21517; 5' GAC-GAA-AGA-GCA-GCC-ATA-GC 3'. The primer pairs 21515/21516 and 21515/21517 were evaluated using a ten-fold dilution series of infected and healthy nucleic acids from *C. quinoa.*

Self-Sustained Sequence Replication (3SR)

3SR amplification was carried out using an adaptation of the method of Fahy *et al* (1991) with two primers, the sequences of which are as follows; 93C104 = 5' AGT-AAT-TTA-ATA-CGA-CTC-ACT-ATA-GGG-AAC-TCG-GCA-TAC-TAT-TCA-CTT 3'; 93C105 = 5' CGA-TTG-GTA-TGA-GTG-ATT-TA 3'.The primers were designed to amplify a specific 500 base pair product from RNA2. 3SR amplifications were carried out using total reaction volumes according to Fahy et al (1991) or 25µl. Units of RNAse-H were as specified by Fahy *et al* (1991) or increased two-fold. Dot blot hybridisation of 3SR products was carried out essentially according to the method of Lair *et al* (1994) using Du Pont nylon Gene Screen Plus membrane. Sample volumes were derived from the Gene Screen Plus methodology. A digoxigenin-dUTP labelled probe produced according to the method of Henry *et al* (1995) was hybridised to nylon blots prepared using a Bio Rad Dot Blot

apparatus. Membrane development was carried out using a Boehringer Non-radioactive Labelling kit.

Immunocapture RT-PCR

The use of IC RT-PCR for the detection of BNYVV in infected sugar beet roots was investigated using a microplate method based on that of Wetzel *et al* (1992) and a single tube method based on that of Jansen *et al* (1990). In the microplate method flat-bottomed microtitre plates were coated with 100µl of polyclonal antibody H3 or monoclonal antibody MAFF9 diluted to 1µg/ml in ELISA coating buffer. Plates were incubated for 3h at 33°C, then washed 3 times with PBS (phosphate-buffered saline). Root samples were prepared by grinding 1:10 (w:v) in 0.005M PO_4 buffer pH7.1 and added to duplicate coated wells of the microtitre plate. Plates were incubated at 4°C overnight then washed with PBS. A multichannel pipette was used to avoid cross-contamination between wells. Plates were washed as described for ELISA (Henry *et al*, 1995), with a total of 4 washes. After washing, 10µl of 1% Triton X-100 solution heated to 65°C was added. Plates were then vortexed for 1min at medium speed to disrupt viral particles using a platform designed to take 96 well plates. The resulting solutions from duplicate wells were pooled to give 20µl of extract per sample which was then used for RT-PCR.

Gel electrophoresis of PCR products

PCR products were analysed by electrophoresis through 1% agarose gels in Tris-borate-EDTA buffer (Tris-borate 22mM, 0.5mM EDTA, pH8). A Pharmacia 100 base pair ladder was used as a size standard together with amplified plasmid pB2 (pB2 contains a fragment of 1-2715 base pairs of RNA2). Gels were stained after electrophoresis with ethidium bromide (0.5µg/ml) and examined on a CAMAG UV transilluminator.

TAS-ELISA and Amplified ELISA

These were carried out as described in Henry *et al* (1995).

RESULTS

Immunocapture RT-PCR

Both methods used produced the expected specific 500 base pair product (Figure 1), the specificity of which was confirmed by sequencing (119 base pairs gave 100% homology with the published sequence) and restriction enzyme digestion. The use of the microplate method generally gave a sensitivity 10 times better than the tube method. A comparison of the trapping efficiency of a polyclonal antiserum (H3) and a monoclonal antibody (MAFF9) showed no difference in sensitivity. Trapping using H3 was subsequently used. Optimisation of the method suggested that increasing the number of PCR cycles from 25 to 40 maximised sensitivity, however other variables such as volume of template made little difference. In general, IC RT-PCR did not produce consistent results and therefore requires much

Figure 1. Sensitivity of IC RT-PCR to detect BNYVV using the microplate method with 40 cycles of amplification and H3 as the trapping antibody. The arrow indicates the 500 base pair PCR product. Lanes 1 and 2. healthy sugar beet root 1:10 and 1:100 dilutions respectively: Lanes 3 to 10. BNYVV infected sugar beet root (dilutions as follows : Lane 3. 10^{-1}: Lane 4. $2x10^{-2}$: Lane 5. 10^{-2}: Lane 6. $2x10^{-3}$: Lane 7. 10^{-3}: Lane 8. $2x10^{-4}$: Lane 9. 10^{-4}: Lane 10. 10^{-5}): Lanes 11 and 12. buffer blank IC-PCR control: Lane 13. sterile distilled water control: Lane 14. *C. quinoa* positive control RNA : Lane 15. Pharmacia 100 bp ladder.

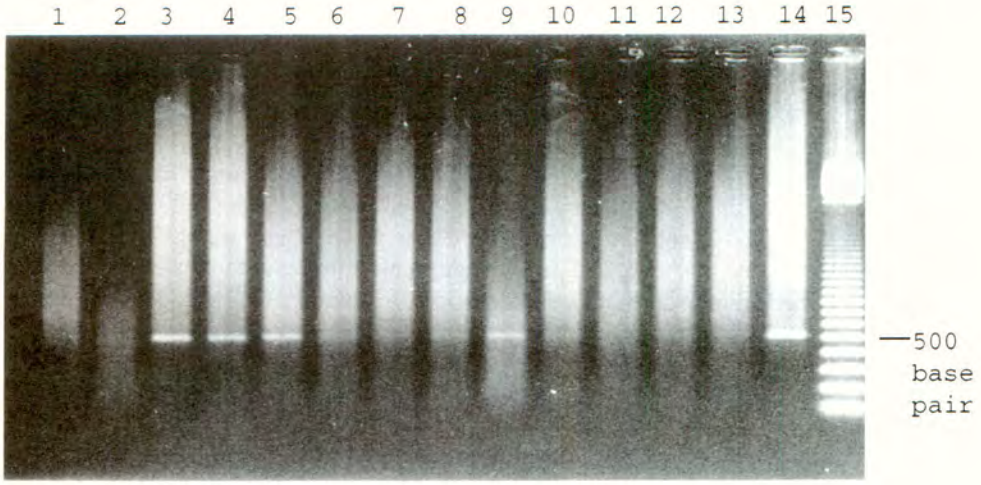
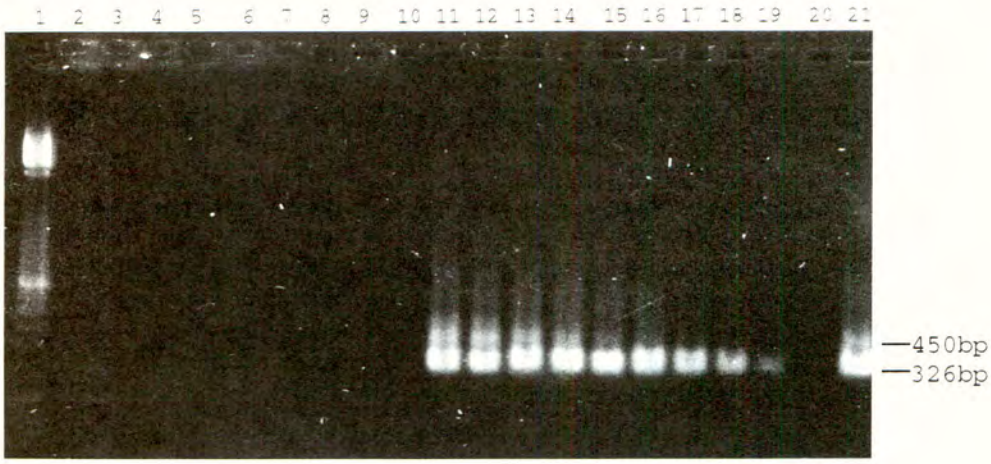


Figure 2. Sensitivity of detection of BNYVV in *C.quinoa* extracts using RT-PCR with nested primers and a cycle of 20+20. The arrow indicates the 326 base pair PCR product. Lane 1. Pharmacia 100 bp ladder.Lanes 2-10. healthy *C. quinoa* extracts (Lane 2. $2x10^{-2}$: Lane 3 . $2x10^{-3}$: Lane 4. $2x10^{-4}$. Lane 5. $2x10^{-5}$: Lane 6. $2x10^{-6}$: Lane 7. $2x10^{-7}$: Lane 8. $2x10^{-8}$: Lane 9. $2x10^{-9}$: Lane 10. $2x10^{-10}$): Lanes 11-19. extracts from *C. quinoa* infected with BNYVV. dilutions as for healthy Lanes 2-10: Lane 20. sterile distilled water: Lane 21. amplified pB2 plasmid control.



improvement before it could be used as a diagnostic test. IC RT-PCR was also 20 times less sensitive than the RT-PCR test for detection of BNYVV in sugar beet roots (Table 1).

3SR

Amplification of infected nucleic acid extracts from *C. quinoa* yielded a specific product of 500 base pairs in size. Dot blot hybridisation of this product confirmed its viral origin. Using the 3SR method it was possible to obtain a product from dilutions of 10^{-2} and 10^{-3} of the extracts (Table 1). Therefore, RT-PCR was found to be 10,000 fold more sensitive than 3SR using an identical extract from *C. quinoa*. Increasing the units of RNAseH and AMV reverse transcriptase in the enzyme mix had no effect on sensitivity. Use of an alternative extraction method (Mumford, 1994) also had no effect. Reduction of the total reaction volume to 25µl produced a negative result. Use of a Qiagen 'RNeasy' RNA extraction kit on 3SR amplified products decreased the amount of debris visualised by gel electrophoresis of the 3SR product but also decreased the amount of the specific 500 base pair product. Detection of the 3SR product using gel electrophoresis and dot blot hybridisation produced similar results in terms of sensitivity.

RT-PCR using nested primers

Initial evaluation of nested primer pairs 21515/21516 and 21515/21517 using 25 cycles of RT-PCR produced the expected products of 318 base pairs and 326 base pairs respectively, for both nucleic acid extracts and pB2 controls. Further evaluation of these primer pairs using the nested PCR method resulted in a number of products specific to infected nucleic acid extracts. These were most numerous for primer pair 21515/21516 using 25+25 cycles of PCR. Primer pair 21515/21517 produced a more specific result with only one additional product of 450 base pairs present using the same number of cycles. Using primer pair 21515/21517, nested PCR consistently detected BNYVV in infected extracts from *C. quinoa*. The expected product of 326bp was produced along with the 450bp product which was absent from more dilute extracts. Using 15+15 cycles or 16+16 cycles of PCR did not improve the result, compared to 25+ 25 cycles. A 20 + 20 cycle PCR proved most sensitive, giving detection down to a dilution of $2x10^{-10}$, and representing a 10,000 fold improvement in sensitivity over a 20 cycle PCR of the same extract.

Table 1 Comparison of the sensitivity of ELISA and nucleic acid amplification methods for the detection of BNYVV in a dilution series of BNYVV infected sugar beet roots and *C. quinoa* leaf sap.

Method	Sensitivity (highest dilution detected)	
	Sugar beet root sap	*C. quinoa* leaf sap
TAS-ELISA	$2.5x10^{-3}$	$1x10^{-4}$
Amplified TAS-ELISA	$4x10^{-4}$	$4x10^{-4}$
RT-PCR	$2x10^{-6}$	$2x10^{-7}$
IC RT-PCR	$1x10^{-5}$	ND
3SR	ND	$2x10^{-3}$
RT-PCR with nested primers	ND	$2x10^{-10}$

DISCUSSION

A comparison of four nucleic acid amplification techniques was done for the detection of BNYVV. Results (Table 1) show that 3SR and immunocapture RT-PCR were generally less sensitive than RT-PCR, whereas RT-PCR using nested primers was ≤ 1000 times more sensitive than the standard RT-PCR. In sensitivity terms, therefore RT-PCR using nested primers looks the most promising test. For diagnostic use, IC RT-PCR would be more acceptable than RT-PCR since the use of immunological trapping avoids the use of hazardous chemicals for RNA extraction, however this technique as it stands is not only less sensitive than RT-PCR but results tend to be inconsistent as well. Further development of the technique to make it more reliable and sensitive may be possible using better antibodies and blotting agents, better viral disruption techniques, RNAse inhibitors, etc. An obvious further development to harness both advantages would be to use IC RT-PCR with nested primers.

ACKNOWLEDGEMENTS

This work was supported by UK Ministry of Agriculture, Fisheries & Food (MAFF) funding and was carried out under MAFF Licence No. PHF 1526A/1233/104.

REFERENCES

Bouzoubaa, S; Ziegler, V; Beck, D; Guilley, H; Richards, K; Jonard, G (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA-2. *Journal of General Virology.* **67**,1689-1700.

Fahy, E; Kwoh, D Y; Gingeras, T R (1991). Self-sustained sequence replication. An isothermal transcription-based amplification system alternative to PCR. *PCR Methods and Applications.* 25.

Henry, C M; Barker, I; Morris, J; Hugo, S A (1995). Detection of beet necrotic yellow vein virus using reverse transcription and polymerase chain reaction. *Journal of Virological Methods.* **54**,15-28.

Jansen, R W; Siegl, G; Lemon, S M (1990). Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proceedings of the National Academy of Science, USA.* **87**, 2897-2871.

Lair, S V; Mirkov, T E; Dodds, J A: Murphy, M F (1994). A single temperature amplification technique applied to the detection of citrus tristeza viroid RNA in plant nucleic acid extracts. *Journal of Virological Methods.* **47,** 141-152.

Liu, S T (1991). Rapid detection of hog cholera virus in tissues by the polymerase chain reaction. *Journal of Virological Methods.* **35**, 227-236.

Mumford, R A; Barker, I; Wood, K R., (1994). The detection of tomato spotted wilt virus using the polymerase chain reaction. *Journal of Virological Methods.* **39**, 27-37.

Tamada, T; Baba, T (1973). Beet necrotic yellow vein virus from Rhizomania affected sugar beet in Japan. *Annals of the Phytopathological Society of Japan.* **39,** 325-332.

Wetzel, T; Candresse, T; Macquaire, G; Ravelonandro, M; Dunez, J (1992). A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. *Journal of Virological Methods.* **39,** 27-37.

DEVELOPMENT OF A SPECIES-SPECIFIC PRIMER FOR *PYTHIUM VIOLAE*

P-H WANG
Microbiology Department, Soochow University, Taipei, 11102, Taiwan, ROC

J G WHITE
Horticulture Research International, Wellesbourne, Warwick, CV35 9EF, UK

ABSTRACT

The internal transcribed spacer (ITS) 1 region of the nuclear ribosomal repeat unit from thirty-six *Pythium* species was amplified by PCR and sequenced. A *Pythium violae*-specific primer Pv1 was identified by sequence analysis of the ITS 1 regions. Primer Pv1 used with the universal primer ITS2 amplified a 136 bp fragment from genomic DNA of *P. violae*, but did not produce a product with DNA from any of 35 other *Pythium* species. The use of PCR amplification of a fragment from the ITS 1 region as a highly specific method for the identification of *P. violae* is reported.

INTRODUCTION

Pythium violae Chesters & Hickman is a soilborne pathogen of considerable importance and is found mainly in temperate regions. The fungus causes most outbreaks of cavity spot of carrot in the UK, most Western European countries, and other countries such as Israel, Australia, Canada and the USA (White, 1988). The only practical control measure for this serious disease is the use of metalaxyl (White, 1988). To avoid prophylactic applications of fungicide, a diagnostic test based on a polyclonal antiserum was developed (White *et al.*, these proceedings). The serological techniques for routine testing of soil are time-consuming, thus, there exists the need for a rapid method of detection of the carrot cavity spot pathogen. The polymerase chain reaction (PCR) was chosen as a possible alternative detection method.

There has been very rapid development of nucleic-acid-based detection techniques. Many of the primers have been developed from the rDNA sequences (Chen, 1994; Ersek *et al.*, 1994; Henson, 1992; Henson *et al.*, 1993). Primer selection from rDNA genes and the spacers between them has certain advantages for fungal identification and detection. The rDNA generally exists in high copy number. This feature provides the sensitivity sufficient for detection, and allows for easier detection in preparations where DNA is in very low concentrations, or where pathogen DNA is a small fraction of the total DNA, e.g. a mixture of fungal and plant DNA from diseased plant extracts. The rDNA region usually consists of three genes, the large subunit gene, the small subunit gene and the 5.8S gene, separated by internal transcribed spacer (ITS) 1 and 2 regions. The ITS regions are useful targets because they appear to be conserved within species, but can vary sufficiently among several plant pathogenic species to allow the identification of unique primer sequences (Chen *et al.*, 1992; Martin; Kistler, 1990; Nazar *et al.*, 1991; Xue *et al.*, 1992). The ITS regions are small enough to be easily generated by PCR and are flanked by highly conserved sequences for which

universal primers have been identified (White *et al.*, 1990). An ideal situation for the application of PCR in diagnostic test and disease epidemiology is that in which DNA sequences exist which are unique to a given pathogen. We have therefore developed a highly specific method for the identification of *P. violae* based on amplification of a fragment from the ITS 1 region by PCR. This was achieved by sequencing the ITS 1 region for thirty-six *Pythium* species (unpublished). The nucleotide sequence variation observed has provided information on interspecies variability within *Pythium*. The species-specific primer was designed from these sequences.

MATERIALS AND METHODS

Fungal cultures

Thirty-six isolates of different *Pythium* species were obtained from the collections of HRI, IMI, and Taiwan (Table 1). Isolate IPV1 served as the DNA sequencing and primer selection isolate for *P. violae*. Isolates were identified or confirmed to species level according to the descriptions and key of Plaats-Niterink (1981), and maintained on cornmeal agar slants at 15°C.

An active culture of each isolate was produced on V-8 agar with incubation at 25°C. After 2 or 3 days, actively growing hyphal regions were cut, and four agar blocks were placed into 200 ml of liquid medium (10 g sucrose, 1 g yeast extract, 1 l DW) (Chen, 1992) in 500 ml flasks, incubated for 3 (fast-growers, e.g. *P. aphanidermatum*) to 42 days (slow-growers, e.g. *P. polymastum*) at 15-30°C. Some *Pythium* species have optimum temperatures as low as 15°C (e.g. *P. violae*), and others are known with optima as high as 35°C (e.g. *P aphanidermatum*, *P. deliense*, and *P. myriotylum*). The mycelia were filtered through two layers of cheesecloth, and then blotted dry with paper towels. The mycelial mat was stored at -70°C until it was used for DNA extraction.

DNA extraction

DNA was extracted from mycelium using a method based on CTAB extraction (Doyle; Doyle, 1990). Dichloromethane was substituted for chloroform in the extraction procedure. Two grams of frozen mycelial mat of each culture were ground to a fine powder in liquid nitrogen with a precooled mortar and pestle. After one extraction with CTAB extraction buffer (1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 2% PVP-40 [w/v]) and two extractions with dichloromethane/isoamyl alcohol (24:1), DNA was precipitated by the addition of isopropanol and centrifuged at 460 **g** for 2 min. The DNA pellet was rinsed twice with 10 ml wash buffer (76% ethanol and 10 mM ammonium acetate) and then spun at 1,500 **g** for 10 min. The pellet was resuspended in 0.5 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 10 µl RNase was added and tubes were incubated overnight at 37°C. The sample was amended with 30 µl 3M Na^+acetate (pH 7.0) and 1 ml ice-cold absolute ethanol, centrifuged for 10 min to precipitate DNA, then dried and resuspended in 200 to 500 µl water (volume depended on yield) overnight at 4°C. DNA was quantified with a fluorometer as instructed by the manufacturer and stored at -20°C.

Table 1. Isolates of *Pythium* used in this study.

Species	Source[a]	Isolate No.	Origin	Host/Habitat
P. acanthicum	HRI	S167	England	*Malus pumila*
P. aphanidermatum	HRI	PA1	England	*Daucus carota*
P. arrhenomanes	HRI	P. arr	England	*Daucus carota*
P. aristosporum	YSL	P37	Taiwan	*Cucumis melo*
P. butleri	HRI	141749	India	*Atropa belladonna*
P. catenulatum	HRI	JW	England	*Rorippa nasturtium-aquaticum*
P. coloratum	IMI	181938	New Zealand	*Daucus carota*
P. deliense	HRI	342678	France	*Persea americana*
P. dimorphum	YSL	P33	Taiwan	*Euphoria longan*
P. dissotocum	IMI	328402	England	*Brassica oleracea*
P. graminicola	HRI	P. gram	England	-
P. heterothallicum	HRI	S4	England	*Malus pumila*
P. hydnosporum	YSL	P294	Taiwan	*Brassica* sp.
P. inflatum	IMI	308148	-	-
P. intermedium	HRI	COM3	England	*Daucus carota*
P. irregulare	YSL	P263	Taiwan	*Allium fistulosum*
P. mamillatum	HRI	P. mam	England	*Daucus carota*
P. myriotylum	YSL	P130	Taiwan	*Brassica chinensis*
P. nunn	IMI	324024	USA	Soil
P. oligandrum	HRI	P. olig	England	*Daucus carota*
P. paroecandrum	HRI	P. paro	England	*Rorippa nasturtium-aquaticum*
P. polymastum	HRI	P. poly	England	*Brassica oleracea*
P. rostratum	HRI	P. ros	England	*Daucus carota*
P. salpingophorum	HRI	L50	England	*Lactuca sativa*
P. spinosum	HRI	P. spino	England	*Daucus carota*
P. splendens	HRI	S138	England	*Malus pumila*
P. sulcatum	HRI	LMW1	England	*Daucus carota*
P. sylvaticum	HRI	OI	England	*Allium cepa*
P. torulosum	YSL	P16	USA	-
P. tracheiphilum	HRI	2A	Spain	*Lactuca sativa*
P. ultimum var. *ultimum*	HRI	PU	England	Soil
P. u. var. *sporangiiferum*	IMI	308275	England	Soil
P. vanterpoolii	HRI	S172	England	-
P. vexans	IMI	132189	Netherlands	*Lycopersicon esculentum*
P. violae	HRI	IPV1	Israel	*Daucus carota*
P. volutum	IMI	331766	Japan	*Triticum* sp. *and Hordeum* sp.

[a]HRI, Horticulture Research International, Wellesbourne, Warwick, UK; IMI, International Mycological Institute, Egham, Surrey, UK; YSL, collection of Y S Lin, Plant Pathology Department, National Chung-Hsing University, Taichung, Taiwan.

DNA amplification and sequencing

Amplifications of the ITS regions of *Pythium* species were performed in a programmable thermal cycler (OmniGene, Hybaid). The primers used were the universal primers ITS1 and ITS2 (White *et al.*, 1990). A 100 µl reaction mixture contained 1.0 µM of each primer, 2.5 units *Taq* DNA polymerase (Boehringer Mannhein), 200 µM each of the four deoxynucleotides, in a PCR buffer, and 0.25 µg of fungal DNA. Reactions were amplified for 30 cycles, DNA denaturation at 94°C, 3 min for the first cycle and 1 min for subsequent cycles, 1 min at 53°C for primer annealing, and 1 min at 72°C for primer extension. For each isolate, at least two independent PCR products were sequenced. Residual primers and dNTPs in the PCR products were removed using the Wizard DNA Clean-Up System Kit (Promega). DNA sequencing was done by the dideoxynucleotide chain termination method of Sanger et al. (1977) with double stranded DNA templates. Sequencing reactions using ITS2 as the sequencing primer were carried out with CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (BioLab). The products from the sequencing reactions were separated by electrophoresis on 7% polyacrylamide gels. The sequence data were analysed by the GCG (Genetic Computer Group software) program version 7-UNIX and aligned manually. Gaps were introduced to the alignment to account for nucleotide insertions of the more divergent taxa. Primer Pv1 (5'GTGTGCGGGACTGGCTGAT3') specific for *P. violae* was selected from variable sections of the ITS 1 region. Primers were synthesized at Genosys Biotechnologies, Inc., Cambridge, UK.

Species-specific amplification of fungal DNA

DNA of thirty-six *Pythium* species isolates was amplified with primers Pv1 and ITS2. At the annealing temperatures of 60°C, 62°C, 64°C, 65°C, and 67°C, the PCR product of target DNA was obtained sufficiently for detection from the gel (data not shown). The PCR mixtures were amplified at a higher stringency, DNA denaturation at 94°C, 3 min for the first cycle and 1 min for subsequent 34 cycles, 20 sec at 67°C for primer annealing, 5 sec at 72°C for extension, and a final cycle of 2 min at 72°C. The reaction took about 130 min. Other PCR conditions were as described above. PCR amplifications containing no DNA template were carried out in every experiment as controls to test for the presence of contamination of reagents and reaction mixtures by nonsample DNA. Efficiency of amplification was monitored by running 8 µl of each reaction through a 1.5% composite agarose (SeaKem GTG, and NuSieve GTG, FSC) gel at 125 V for 3 hr in Tris-borate-EDTA buffer (0.44 M boric acid, 0.44 M Trizma base, and 10 mM EDTA). A 100-bp molecular weight ladder (Pharmacia) was used as the size standard, stained with ethidium bromide, with visualization and photography under ultraviolet light.

RESULTS

Selection of primer

The ITS 1 region of the rDNA of *Pythium* species was initially amplified and directly sequenced with the universal primers ITS1 and ITS2. The sequence data from the isolates of 36 *Pythium* species showed different level of homology between species (unpublished). The ITS 1 region of *P. violae* was shown to be 182 bp. The primer Pv1 (19 nucleotides) was

selected from a variable region 80 bp downstream from the ITS1 primer sequence in *P. violae* (Fig 1).

TGCTGTGCTGTGGTGTTTTTGGACGTGGCTGTGGCGGAGGAGGAACGAAGGTTGGTCTNG 60

TGTGTGCACAGCAATGTGTGTGTGCGGGACTGGCTGATCTATTTTTTTTAAACCCATACA 120

AAATGACTGATTTATACTGTGAGAACGAAAGTTCTTGCTTTTAACTAGATAACAACTTTNAG 182

Fig 1. Nucleotide sequence of the ITS 1 region of *P. violae*. The primer Pv1 is underlined.

Species-specific DNA amplification

The Pv1 and ITS2 primers amplified a single 136 bp fragment from the DNA of *P. violae*. The primers did not amplify genomic DNA from the other 35 *Pythium* species isolates (Fig 2).

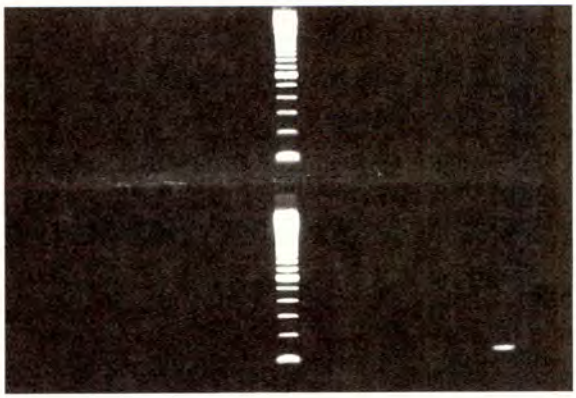


Fig 2. Amplification products from primer pair Pv1-ITS2 with 36 *Pythium* species. A 136 bp DNA fragment was amplified from *P. violae*. Middle lane, molecular size marker (100 bp ladder).

DISCUSSION

Sufficiently stringent PCR conditions were established by increasing the primer annealing temperature and decreasing the extension time to differentiate *P. violae* from other *Pythium* species. The rate and specificity of amplification were influenced strongly by annealing temperature. Both the expected melting temperature of primer Pv1 and ITS2 were 62°C. The optimal annealing temperature of 67°C was selected. Amplification of nonspecific DNA sequences at low stringency might be due to mispriming and/or misextension (Ersek *et al.*, 1994). Our experiments have shown that this modification gives the required specificity.

Sequence analysis of the ITS region and primer selection in *Pythium* species can be used in developing species-specific oligonucleotide primers for other *Pythium* diseases, and could be employed routinely and efficiently in pathogen identification and detection. An assay using PCR can be completed in one day, and is seen to be effective, simple and sensitive.

REFERENCES

Chen, W (1992) Restriction fragment length polymorphisms in enzymatically amplified ribosomal DNAs of three heterothallic *Pythium* species. *Phytopathology*. **82**, 1467-1472.

Chen, W (1994) Development of specific primers for identification and detection of *Pythium arrhenomanes*. *Phytopathology*. **84 (10)**, 1087. (abstract)

Chen, W; Hoy, J W; Schneider, R W (1992) Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythium* species. *Experimental Mycology*. **16**, 22-34.

Doyle, J J; Doyle, J L (1990) Isolation of plant DNA from fresh tissue. *Focus*. **12**, 13.

Ersek, T; Schoelz, J E; English, J T (1994) PCR amplification of species-specific DNA sequences can distinguish among *Phytophthora* species. *Applied and Environmental Microbiology*. **60 (7)**, 2616-2621.

Henson, J M (1992) DNA hybridization and polymerase chain reaction (PCR) tests for identification of *Gaeumannomyces*, *Phialophora* and *Magnaporthe* isolates. *Mycological Research*. **96 (8)**, 629-636.

Henson, J M; Goins, T; Grey, W; Mathre, D E; Elliott, M L (1993) Use of polymerase chain reaction to detect *Gaeumannomyces graminis* DNA in plants grown in artificially and naturally infested soil. *Phytopathology*. **83**, 283-287.

Martin, F N; Kistler, H C (1990) Species-specific banding patterns of restriction endonuclease digested mitochondrial DNA from genus *Pythium*. *Experimental Mycology*. **14**, 32-46.

Nazar, R N; Hu, X; Schmidt, J; Culham, D; Robb, J (1991) Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. *Physiological and Molecular Plant Pathology*. **39**, 1-11.

Plaats-Niterink, A J (1981) *Monograph of the genus Pythium. Studies in Mycology No. 21*. Baarn, Netherlands: Centraal Bureau Voor Schimmelcultures.

Sanger, F S; Nicklen, S; Coulson, A R (1977) DNA sequencing with chain-termination inhibitors. *Proceedings of the National Academy Sciences of the United States of America*. **74**, 5463-5467.

White, J G (1988) Studies on the biology and control of cavity spot of carrots. *Annals of Applied Biology*. **113**, 259-268.

White, T J; Bruns, T; Lee, S; Taylor, J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, A Guide to Methods and Applications*, Innis, M A; Gelfand, D H; Sninsky, J J; White, T J (eds), San Diego: Academic Press, pp. 315-322.

Xue, B; Goodwin, P H; Annis, S L (1992) Pathotype identification of *Leptosphaeria maculans* with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences. *Physiological and Molecular Plant Pathology*. **41**, 179-188.

MOLECULAR CHARACTERIZATION AND DIAGNOSIS OF *COLLETOTRICHUM ACUTATUM*

S SREENIVASAPRASAD[1], K SHARADA[2], A E BROWN[2], P R MILLS[1]
[1]Horticulture Research International, Wellesbourne, Warwick CV35 9EF
[2]Department of Applied Plant Science, The Queen's University of Belfast, Belfast BT9 5PX

ABSTRACT

Nucleotide sequence of the rDNA internal transcribed spacer (ITS) 1 region of 19 isolates of *Colletotrichum* identified as *C. gloeosporioides* or *C. musae* was determined. High homology (98-100%), of these isolates to previously characterised *C. acutatum* isolates and only 79-81% homology to *C. gloeosporioides/C. musae* enabled their identification as *C. acutatum*. A *C. acutatum*-specific primer (*Ca*Int2) was designed from the variable region of ITS1. Diagnostic PCR with *Ca*Int2 and ITS4, a universal primer, enabled accurate identification of *C. acutatum* cultures and detection of the pathogen in infected strawberry tissue.

INTRODUCTION

The genus *Colletotrichum* includes several important phytopathogens. Despite efforts to develop proper species concepts, the phenotypic plasticity of several *Colletotrichum* species has made their correct identification difficult and ambiguous (Sutton, 1992).

Clarification of species concepts in *Colletotrichum* is a matter of considerable practical importance not only for identification, but also to assemble precise data on host range and geographic distribution. This information is essential in disease diagnosis and control, quarantine and trade. For example, at present, it is impossible to know in many cases which taxon precisely is being referred to under the name *C. gloeosporioides*.

In view of the unreliability of the conventional taxonomic characteristics, we are investigating the potential of various molecular markers for understanding the systematics and phylogeny of *Colletotrichum* species and for their diagnosis (Sreenivasaprasad *et al.*, 1993, 1994, 1996a, 1996b; Mills *et al.*, 1992; Brown *et al.*, 1996). It is in this context that we analysed strains of *Colletotrichum* from lupin, citrus and rubber which were all described as *C. gloeosporioides* (R Cook, pers comm; Agostini *et al.*, 1992; Brown; Soepena, 1994). The morphological descriptions of these strains, though not typical, were reminiscent of *C. acutatum.* Similar strains have also been observed on stone and pome fruits, apple, walnut and *Xanthium* (Walker *et al.*, 1991).

C. acutatum was described by Simmonds (1965, 1968) from Australia on papaya, tomato

and strawberry. *C. acutatum* is slower-growing than *C. gloeosporioides* and produces narrowly fusiform conidia as against the straight, cylindrical conidia of the latter. The known host range and geographic distribution of *C. acutatum* has considerably extended; is now recognised on 34 hosts from 22 botanical families and has been reported from countries on all continents (Walker *et al.*, 1991).

C. acutatum is the causal agent of anthracnose of strawberry in the UK and continental Europe. *C. fragariae* and *C. gloeosporioides* also incite the disease in the USA. *C. acutatum*-infected plants have been brought unwittingly into the UK in the past, consequently all imported consignments are now tested for blackspot at the Central Science Laboratory (MAFF), Harpenden. Rapid and sensitive diagnostic tests have been sought to detect the symptomless phase of *C. acutatum* on imported plants.

Use of rDNA sequence data for accurate identification of *C. acutatum* and PCR-based diagnosis of the pathogen in plant tissue are described.

MATERIAL AND METHODS

Fungal cultures and DNA extraction

Details of the isolates used are given in Table 1. Cultures were maintained on potato dextrose agar at 25°C. Fungal mycelium was produced in glucose casamino acid liquid medium. DNA was extracted from freeze-dried mycelial powder (Sreenivasaprasad *et al.*, 1992).

Nucleotide sequence determination of the internal transcribed spacer (ITS)1 region of the rDNA was carried out using manual and automated sequencing technologies. For manual sequencing, the ITS1 region was amplified using primers ITS1 and ITS2 (White *et al.*, 1990) at 55°C annealing temperature. PCR conditions and solid phase sequencing methodology were as described in Sreenivasaprasad *et al.* (1992). ITS1 and ITS2 were used as the sequencing primers with the T7 DNA sequencing kit (Pharmacia).

For automated sequencing, the ITS1 region was amplified using primers ITS1ext (GTAACAAGGTTTCCGTAGGTG) and ITS2ext (ATTTCGCTGCGTTCTTCATCG). PCR conditions were as described in Sreenivasaprasad *et al.* (1992), with 55°C annealing temperature. Purified PCR products (Wizard Clean-up System, Promega) were used for cycle sequencing. Reactions were performed on a thermal cycler using 500 ng of template DNA and the Prism ready reaction dyedeoxy terminator kit (Applied Biosystems). The transcripts were electrophoresed in an automated sequencing apparatus (373A DNA sequencing system, Applied Biosystems). Reactions and electrophoresis were performed according to manufacturer's instructions.

The nucleotide sequences were read and edited using 373A gene scan software and sequence navigator (Applied Biosystems), respectively. The sequence data was managed

on DNASIS (LKB) and aligned and analysed on CLUSTAL V package (Higgins *et al.*, 1992).

DNA extraction from plants

DNA extraction from freeze-dried and fresh strawberry tissue was based on Torres *et al.* (1993). To 50 mg dry powder or 100 mg fresh tissue, 1 ml of 2X CTAB buffer was added. Samples were incubated at 65°C for 30 min after thorough mixing and addition of 100 µl chloroform and octanol (24:1). Samples were cooled to room temperature and approx. 750 µl of chloroform/octanol were added and thoroughly mixed. The aqueous phase was collected by centrifugation (14,000 rpm, 10 min) and DNA precipitated with 2 volumes of absolute ethanol and washed with 76% ethanol containing 0.2 M sodium acetate. DNA was dissolved in 200 µl TE, mixed with 25 µl RNase (1 mg ml^{-1} stock) and incubated at 37°C for 30 min. After neutral phenol extraction (twice), DNA was precipitated with absolute ethanol, washed twice with 70% ethanol and dissolved in 100 µl TE.

Diagnostic PCR

PCR mixtures (100 µl) contained genomic DNA (50-100 ng), PCR buffer (Promega), 200 µM of each dNTP, 0.5 µM each of primers *Ca*Int2 and ITS4 and 2.5 units of *Taq* DNA polymerase. Reactions were subjected to 30 cycles of 1.5 min at 94°C, 2 min at 55°C and 3 min at 72°C. Ten to 20 µl of the PCR products were checked on agarose gels (1.4%, w/v) by ethidium bromide staining (0.5 µg ml^{-1}).

RESULTS AND DISCUSSION

Multiple sequence alignment and analysis revealed that the 19 *Colletotrichum* isolates, originally described as *C. gloeosporioides* or *C. musae,* showed 98-100% homology (0-2% variation) to the reference *C. acutatum* isolates 397 and NI 90 (Table 1) and fitted well within the range of variation (0-6%) established in this species (Sreenivasaprasad *et al.*, 1992). An isolate from banana, supplied as *C. musae*, showed only 79% homology to a reference *C. musae* isolate (Sreenivasaprasad *et al* 1994). The other 18 isolates, identified as *C. gloeosporioides,* showed only 80-81% homology to the named species. This clearly identifies these isolates as *C. acutatum.*

Having established the intra-specific divergence and inter-specific homology levels in ITS1 among 18 species of *Colletotrichum* (Sreenivasaprasad *et al.*, 1996a) including *C. acutatum, C. gloeosporioides* and *C. musae*, it is apparent that the low level of homology (79-81%) shown by these 19 isolates to isolates of their originally named species is indicative of the elasticity of the species concepts based on characters such as conidial shape and size and colony morphology which are highly variable.

Table 1. ITS1 sequence homology (%) of 19 *Colletotrichum* isolates to reference *C. acutatum* isolates (397 and NI 90)

No. of isolates	Host	Original identification	Homology to named species (%)	Homology to *C. acutatum* (%)
7	Lupin	*C. gloeosporioides*	80	99
4	Sweet orange	*C. gloeosporioides*	80-81	99-100
4	Key lime	*C. gloeosporioides*	80-81	99-100
2	Rubber	*C. gloeosporioides*	81	100
1	Phormium	*C. gloeosporioides*	81	98
1	Banana	*C. musae*	79	100

The ITS1 sequence appeared a suitable target for designing a *C. acutatum*-specific primer for diagnostic purposes. Hence the primer *Ca*Int2 (GGGGAAGCCTCTCGCGG) was designed from the variable region of *C. acutatum,* based on a multiple sequence alignment of a number of *Colletotrichum* spp. (Sreenivasaprasad *et al.*, 1996a).

Primer *Ca*Int2 in conjunction with ITS4 amplified a fragment of 490 bp from all the isolates tested along with the reference *C. acutatum* isolates 397 and NI 90 (Fig 1). The primer was specific to *C. acutatum*; no amplification product was obtained with genomic DNA from other *Colletotrichum* spp. (all of which tested positive with universal primers). On the other hand, none of the test isolates gave an amplification product with primer *Cg*Int specific for *C. gloeosporioides* (data not shown).

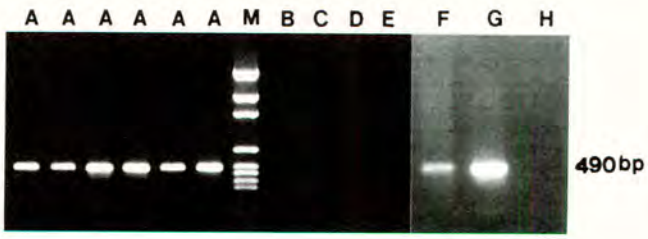


Fig 1. Amplification of a *C. acutatum*-specific product using primers *Ca*Int2 and ITS4. Lanes A, *C. acutatum*; B, *C. linicola*; C, *C. graminicola*; D, *C. gloeosporioides*; E, *C. fragariae*; F, infected strawberry leaf tissue; G, infected strawberry fruit tissue; H, healthy strawberry leaf tissue.

Primers *Ca*Int2 and ITS4 used in PCR with total DNA extracted from strawberry leaves infected with *C. acutatum* and from blackspotted fruit amplified a product identical in size (490 bp) to that amplified from fungal DNA. Hybridisation analysis of the product from strawberry tissue indicated its fungal origin (data not shown).

PCR amplification of serial dilutions of a known quantity of *C. acutatum* DNA and infected strawberry DNA indicated that approx. 100 fg of fungal DNA, representing 1 ng of fungal mycelium, could be detected. Successful detection of *C. acutatum* in strawberry tissues was dependent on the method of DNA extraction.

The diagnostic PCR test developed could be used for direct detection of *C. acutatum* on host plants other than strawberry, and to accurately identify this ubiquitous and highly variable pathogen.

REFERENCES

Agostini, JP;Timmer, LW; Mitchell, DJ (1992) Morphological and pathological characteristics of strains of *Colletotrichum gloeosporioides* from citrus. *Phytopathology*. **82**,1377-1382.

Brown, AE; Soepena, H (1994) Pathogenicity of *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* on leaves of *Hevea* spp. *Mycological Research*. **98**, 264-266.

Brown, AE; Sreenivasaprasad, S; Timmer LW (1996) Molecular characterisation of slow-growing orange and Key lime anthracnose strains of *Colletotrichum* from citrus as *C. acutatum*. *Phytopathology* (in press).

Higgins, DG; Bleasby, AJ; Fuchs, R (1992) CLUSTAL V: improved software for multiple sequence alignment. *CABIOS* **8**. 189-191.

Mills, PR; Sreenivasaprasad, S; Brown, AE (1992) Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. *FEMS Microbiology Letters*. **98**, 137-144.

Simmonds, JH (1965) A study of the species of *Colletotrichum* causing ripe fruit rots in Queensland. *Queensland Journal of Agricultural and Animal Sciences*. **22**, 437-459.

Simmonds, JH (1968) Type specimens of *Colletotrichum gloeosporioides* var. *minor* and *Colletotrichum acutatum*. *Queensland Journal of Agricultural and Animal Sciences*. **25**, 178A.

Sreenivasaprasad, S; Brown, AE; Mills, PR (1992) DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. *Physiological* and *Molecular Plant pathology*. **41**, 265-281.

Sreenivasaprasad, S; Brown, AE; Mills, PR (1993) Coffee berry disease pathogen in Africa: genetic structure and relationship to the group species *Colletotrichum gloeosporioides*. *Mycological Research*. **97**; 995-1000.

Sreenivasaprasad, S; Mills, PR; Brown AE (1994) Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum. Mycological Research.* **98**, 186-188.

Sreenivasaprasad, S; Mills, PR; Meehan, B; Brown, AE (1996a) Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. *Genome* (in press).

Sreenivasaprasad, S; Sharada, K; Brown, AE; Mills, PR (1996b) PCR-based detection of *Colletotrichum acutatum* on strawberry. *Plant Pathology* (in press).

Sutton, BC (1992) The genus *Glomerella* and its anamorph *Colletotrichum.* In: Colletotrichum - *Biology, Pathology and Control,* Bailey, JA; Jeger MJ (eds), Wallingford: CAB International, pp. 1-26.

Torres, AM; Weeden, NF; Martin, A (1993) Linkage among isozyme, RFLP and RAPD markers in *Vicia faba. Theoretical and Applied Genetics.* **85**, 937-945.

Walker, J; Nikandrow, A; Millar, GD (1991). Species of *Colletotrichum* on *Xanthium* (Asteraceae) with comments on some taxonomic and nomenclatural problems in *Colletotrichum. Mycological Research.* **95**, 1175-1193.

White, TJ; Bruns, T; Lee, S; Taylor, J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, A Guide to Methods and Applications.* Innis, MA, Gelfand, DH; Sninsky, JJ; White TJ (eds), San Diego: Academic Press, pp. 315-322.

DIAGNOSTIC PCR FOR *TRICHODERMA HARZIANUM* (GROUP 2), AN AGGRESSIVE COLONISER OF MUSHROOM COMPOST

S. MUTHUMEENAKSHI & P. R. MILLS
Department of Microbial Biotechnology, Horticulture Research International, Wellesbourne, Warwick, CV35 9EF

ABSTRACT

Isolates of *Trichoderma harzianum* obtained from mushroom compost could be differentiated into three distinct groups based on RFLP analysis of rDNA and mtDNA, RAPD patterns and sequence data of the internal transcribed spacer region 1 (ITS1). A sequence specific to *T. harzianum* (group 2) was identified within the ITS1 region and used to design a PCR primer (ThI-int). This primer, when used in conjunction with a universal primer from the nuclear large ribosomal gene, amplified a fragment of ~450 bp from aggressive colonising isolates of *T. harzianum* but did not prime amplification from any other *Trichoderma* spp. Preliminary experiments suggest that amplification of this product may be possible following DNA extraction from colonised mushroom compost.

INTRODUCTION

Trichoderma species are associated with various diseases of mushroom such as cap spotting, green mould and blotch (Beach, 1937, Kligman, 1950, Kneebone & Merek, 1959). In 1985-1986, a green mould epidemic devastated parts of the mushroom industry in the British Isles (Seaby, 1987) and losses ran into several million pounds (Fletcher, 1990). A second Ireland-wide green mould epidemic occurred during 1989-1990 (Doyle, 1991) and in 1993, a smaller but significant outbreak occurred (Doyle, Pers. Comm.). Subsequently, the causal agent for 1985-1986 episode was identified as a strain of *T. harzianum* (Seaby, 1987). Of the three biological forms of *T. harzianum* (Th1, Th2 and Th3) recognised on the basis of growth rate and time and pattern of sporulation, strain Th2 was found to be the most aggressive one (Seaby, 1987; Doyle, 1991). However, not all isolates can be reliably identified by these characters and morphologically *T. harzianum* isolated from mushroom compost in the UK conformed to only one group species.

Effective disease management primarily depends on accurate diagnosis of pathogen. Farm hygiene, chemical and environmental control are recommended as disease control measures against *Trichoderma* attacks, where resistant commercial strains of *Agaricus* are unavailable. In this paper, we outline the development of various molecular markers using DNA-based methods for reliable identification of *T. harzianum* (group 2) isolates. The variable spacer region in the rDNA repeat unit is an attractive source for generating species-specific PCR primers (Brown *et al.*, 1993; Mills *et al.*, 1992; Moukhamedov *et al.*, 1994; Nazar *et al.*,

1991; Sreenivasaprasad *et al.*, 1994). We also describe the design of a PCR primer from the ITS 1 of *T. harzianum* (group 2) and identification of *T. harzianum* (group 2) isolates.

MATERIALS AND METHODS

Eighty-one isolates of *T. harzianum* from mushroom compost from Northern Ireland, England and the Republic of Ireland were used in this study. The isolates are listed elsewhere (Muthumeenakshi *et al.*, 1994). All isolates were identified as *T. harzianum* according to the criteria of Rifai (1969). The cultures were maintained on potato dextrose agar at 25°C.

DNA extraction, mitochondrial DNA (mtDNA) isolation, Southern blotting, probe labelling, hybridization, autoradiography and Random Amplified Polymorphic DNA (RAPD) analysis were done as described by Muthumeenakshi *et al.* (1994). For rDNA Restriction Fragment Length Polymorphism (RFLP) analysis, Southern blots were probed with plasmid pMY60 containing a complete rDNA unit from *Saccharomyces carlsbergensis* (Verbeet *et al.*, 1983). For mtDNA RFLP analysis mitochondrial DNA purified from isolate ThI was used as a probe.

The ITS1 region of rDNA was amplified using biotinylated ITS1 and ITS2 primers (White *et al.*, 1990). PCR conditions and solid phase sequencing methodology were as described by Muthumeenakshi *et al.* (1994). The sequences were read manually into DNASIS (LKB/Pharmacia) on a PC. The multiple sequence alignment of the data was carried out using CLUSTAL V (Higgins *et al.*, 1992) from the collection of programs available on the Seqnet Unix System at the Daresbury Laboratory (SERC, Warrington, UK).

RESULTS

RFLP analysis

Ribosomal DNA restriction patterns with *Bam*H I, *Cla* I, *Eco*R I, *Hin*d III, *Pst* I and *Sac* I distinguished 36 isolates of *T. harzianum* (group 2) from *T. harzianum* (group 1) and (group 3) isolates (data not shown). All isolates of *T. harzianum* (group 2) produced identical rDNA restriction enzyme digestion fragments with each of the restriction enzymes used. There was no restriction site in the rDNA detected with *Bam*H I, ~10.2 kb fragment with *Hin*d III, ~9.9 kb fragment with *Pst* I and 3.1 kb fragment (possibly more than one) with *Eco*R I digestion. *Sac* I digestion resulted in two fragments 7.4 kb and 2.0 kb and *Cla* I in three fragments, of size 7.0 kb, 2.2 kb and 1.1 kb. The approximate unit length of rDNA for *T. harzianum* (group 2) isolates was 11-12 kb.

MtDNA RFLP analysis with *Hind* III revealed limited polymorphism and divided the isolates into five subgroups. At least 20 to 23 fragments were generated by *Hind* III digestion, of which 16 were common to all isolates (data not presented). The size of mitochondrial genome of *T. harzianum* (group 2) was calculated to be 60-62 kb.

RAPD analysis

The RAPD analysis was done for ten isolates of *T. harzianum* (group 2) using six primers (A03, A11, A13, B06, B07 and B10; Operon Inc.). All isolates produced an identical pattern with each of five primers and primer B10 differentiated them into two groups on the basis of presence/absence of a single fragment (data not shown).

ITS 1 sequence analysis

Five isolates of *T. harzianum* (group 2) were sequenced and found to be identical. The length of ITS1 was 202 bp (data not shown).

Diagnostic PCR

The ITS1 sequence of *T. harzianum* (group 2) was aligned with ITS1 sequences of *T. harzianum* (group 1) and (group 3) isolates. Sequence alignment is published elsewhere (Mills & Muthumeenakshi, 1994, Muthumeenakshi *et al.*, 1994). A region for a primer specific to *T. harzianum* (group 2) isolates was chosen to generate the primer ThI-int
5' CCCCCTCGCGGGTTATTTTTACT 3'.

The *T. harzianum* (group 2) specific primer ThI-int and a modified universal primer ITS4 (modified from White *et al.*, 1990) were used to amplify a fragment of ~450 bp with DNA from *T. harzianum* (group 2) isolates. The PCR mixtures were subjected to 30 cycles of 1 minutes at 94°C, 2 minutes at 66°C and 2 minutes at 72°C. *T. harzianum* (group 2) isolates amplified a fragment of 450 bp in size whereas *T. harzianum* (group 1) and (group 3) isolates did not (Figure 1).

Our preliminary experiments on detection of aggressive colonising isolates from mushroom compost have given encouraging results. Genomic DNA of *T. harzianum* (group 2) added to DNA extracted from spawn-run mushroom compost (modified from Sonnenberg *et al.*, 1995) was used successfully to amplify the *T. harzianum* (group 2) specific fragment using the PCR conditions specified above.

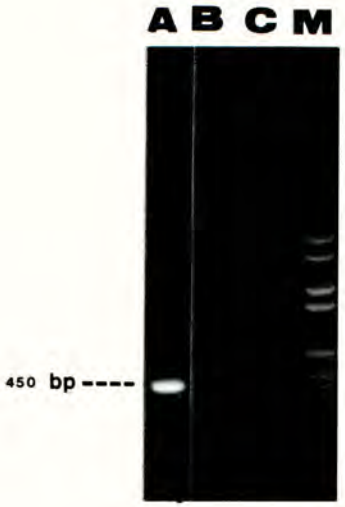


Figure 1 Diagnostic PCR for aggressive isolates of *T. harzianum*.

Lanes: A - *T. harzianum* (group 2); B - *T. harzianum* (group 1); C - *T. harzianum* (group 3) and M refers to DNA molecular weight marker VI (Boehringer Mannheim).

DISCUSSION

The results underline the value of molecular markers in the identification of *T. harzianum* (group 2). Isolates of *T. harzianum* (group 2) were well characterised and reliably identified using molecular markers. Unambiguous differentiation of the aggressive coloniser from morphologically similar but non-aggressive species is essential to assess the risk to the industry. The RFLP and RAPD analyses revealed an estimate of the variation and complexity of the pathogen population. The genetic uniformity in *T. harzianum* (group 2) isolates supports the view that the green-mould outbreaks within the British Isles may have originated from a single source, as yet unidentified.

The PCR primer ThI-int may allow rapid identification of the aggressive coloniser directly from mushroom compost.

ACKNOWLEDGEMENTS

Part of this work was funded by the Horticultural Development Council. SM was partly funded by the Thomas Henry Scholarship, The Queen's University of Belfast. The authors also gratefully acknowledge the contributions made by Dr Averil Brown, Dr John Fletcher and Mr David Seaby.

REFERENCES

Beach, W W (1937) Control of mushroom diseases and weed fungi. *Pennsylvannia State College of Agriculture Bulletin.* **351**, 1-32.

Brown, A E; Muthumeenakshi S; Sreenivasaprasad S; Mills P R; Swinburne, T R (1993) A PCR primer specific to *Cylindrocarpon heteronema* for detection of the pathogen in apple wood. *FEMS Microbiology Letters.* **108**, 117-120.

Doyle, O (1991) *Trichoderma* green mould - update. *Irish Mushroom Review.* **3**, 13-17.

Fletcher, J T (1990) *Trichoderma* and *Penicillium* diseases of *Agaricus bisporus.* A literature review for the Horticultural Development Council, London: ADAS.

Higgins, D G; Bleasby A J; Fuchs R (1992) CLUSTAL V: Improved software for multiple sequence alignment. *Computer Applied BioScience.* **8**, 189-191.

Kligman, A M (1950). Handbook of mushroom culture. J B Swayne (ed.). Kennett Square, Pennsylvannia.

Kneebone, L R; Merek E L (1959) Brief outline of and controls for mushroom pathogens, weed moulds, indicator moulds and competitors. *Mushroom Growers Association Bulletin.* **113**, 146-153 and **114**, 190-193.

Mills, P R; Sreenivasaprasad S; Brown A E (1992) Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. *FEMS Microbiology Letters.* **98**, 137-144.

Mills, P R; Muthumeenakshi S (1994) Identification of colonising forms of Trichoderma species isolated from mushroom compost. In: *Ecology of Plant Pathogens.* J.P. Blakeman & B. Williamson (eds), pp. 135-142.

Moukhamedov, R; Hu X; Nazar R N; Robb J (1994) Use of polymerase chain reaction-amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricarpus*. *Phytopathology.* **84**, 256-259.

Muthumeenakshi, S; Mills P R; Brown A E; Seaby D A (1994) Intraspecific molecular variation among *Trichoderma harzianum* isolates colonising mushroom compost in the British Isles. *Microbiology.* **140**, 769-777.

Nazar, R N; Hu X; Schmidt J; Culham D; Robb J (1991). Potential use of PCR-amplified detection and differentiation of *Verticillium* wilt pathogens. *Physiological and Molecular Plant Pathology.* **39**, 1-11.

Rifai, M (1969) A revision of the genus *Trichoderma*. In: *Mycological Paper 116.* Kew, Surrey, England.

Seaby, D A (1987) Infection of mushroom compost by *Trichoderma* species. *Mushroom Journal.* **179**, 355-361.

Sonnenberg, A S M; van Kempen I P J; van Griensven L J L D (1995) Detection of *Agaricus bisporus* viral dsRNAs in pure cultures, spawn and spawn-run compost by RT-PCR. *Mushroom Science*. **14**, 587-594.

Sreenivasaprasad, S; Mills P R; Brown A E (1994) Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. *Mycological Research*. **98**, 186-188.

Verbeet, M Ph; Klootwijk J; Herrikhuizen H; Fontijn R; Vreugdenhil E; Planta R J (1983) Molecular cloning of the rDNA of *Saccharomyces rosei* and comparison of its transcription initiation region with that of *Saccharomyces carlsbergensis*. *Gene*. **23**, 53-63.

White, T J; Bruns T; Lee S; Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* .M A Innis, D H Gelfand, J J Sninsky & T J White (eds), pp. 315-322. San Diego: Academic Press, Inc.

RAPID DIFFERENTIATION OF CLOSELY RELATED ISOLATES OF ZUCCHINI YELLOW MOSAIC VIRUS BY POLYMERASE CHAIN REACTION AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

N J SPENCE, A MILLER
Horticulture Research International, Wellesbourne, Warwick, CV35 9EF, UK

D J BARBARA and A MORTON
Horticulture Research International, East Malling, West Malling, Kent, ME19 6BJ, UK

ABSTRACT

A method has been developed for discriminating serologically indistinguishable isolates of ZYMV based on immunocapture reverse transcriptase-polymerase chain reaction (RT-PCR) followed by RFLP analysis of the amplified product. Only limited sequence information was available at the time of primer design but most isolates could be amplified. Restriction endonucleases revealing suitable RFLPs were readily identified. Each of two isolates of ZYMV could be detected in the presence of the other and the relative proportions approximately quantified by visual examination of digestion fragments. The method is currently being used to investigate the epidemiology of ZYMV in the field in plants which have been inoculated with the mild strain of ZYMV to cross-protect against severe forms of the virus. The procedure is rapid (it can be completed in less than two days) and effective and will probably be generally applicable to distinguishing closely related isolates, even where little sequence information is available.

INTRODUCTION

Isolates of plant viruses with quite distinct biological effects may be closely related, or even indistinguishable, serologically and this can make rapid differentiation difficult. Biological assays are often slow and laborious or are difficult to apply when two strains are present in the same host plant. This paper describes a rapid procedure capable of differentiating and quantifying mixed isolates. Zucchini yellow mosaic potyvirus (ZYMV) is an important pathogen in cucurbit crops worldwide and is spread by aphid-borne transmission from initial infections probably arising from seed-transmission (Schrijnwerkers *et al.*, 1991). One possibility being investigated for the control of ZYMV in the UK is the use of mild-strain cross-protection (Walkey *et al.*, 1992). In these studies it would be useful to be able to follow the spread and multiplication of both the mild, protecting isolate and the severe,

challenging isolate within the same plant. This is difficult as no means are available of distinguishing most isolates physically or serologically and there is a need to identify and quantify (at least approximately) virus isolates in mixed infections.

Immunocapture reverse-transcriptase PCR (Jansen, Siegl & Lemon, 1990; Wetzel *et al.*, 1992; Lanneau & Candresse pers. comm.) followed by RFLP analysis of the PCR products is here shown to be capable of rapidly (less than 36 hrs) distinguishing isolates of ZYMV and showing the approximate relative amounts of two isolates in mixed infections. Some knowledge of RNA sequences was necessary in order to develop PCR primers; at the time of primer design a small number of partial sequences of ZYMV were available from databases. Several further sequences of ZYMV, including two of the entire virus, are now available.

MATERIALS AND METHODS

Virus isolates and antiserum

ZYMV isolate ZYMV:Wk was a variant that induced mild mottle in melon (*Cucumis melo*) and courgette (*Cucurbita pepo*) leaves and was isolated in 1986 from a poorly aphid-transmissable isolate E15-PAT in France (Lecoq *et al.*, 1991). The severe strain ZYMV:Fa was isolated from courgette plants in a disease outbreak in the Vale of Evesham, UK in 1989. The Reunion isolate (ZYMV:Re) was isolated from courgette in Reunion and provided by H Lecoq. For this work all ZYMV isolates were inoculated to *C. pepo* cv. Goldrush and maintained in a glasshouse prior to use. A polyclonal antiserum to ZYMV was taken from the HRI collection and was effective in ELISA (Walkey *et al.*, 1992). Immunoglobulin was prepared using standard procedures. Total RNA was prepared from virus-infected and control plants using standard methods.

Viral sequences and PCR primers

Published sequences of ZYMV were available and PCR primers were designed manually from these. At the time of primer design several partial, but no full length, sequences were available for ZYMV and these were compared (using GeneJockey II, Biosoft, Cambridge, UK) to try to identify some conserved regions. Two pairs of primers were designed, intended to amplify *c.* 20% of the genome close to the 3' terminus (as overlapping sections of *c* 1200 nts and 1050 nts). Most studies have been with primers 252 and 253.

Primer sequences were:(i) 250/5'-CGC AGT GCT CAT AAT CAG GTC G-3' (upstream, RNA sense; nts 7777-7798) and 251/5'-GCT GAT GAG ACG CTC GTG TG-3' (downstream, antisense; nts 8821-8840); (ii) 252/5'GCT CCA TAC ATA GCT GAG AC-3' (upstream, RNA sense; nts 8408-8427) and 253/5'-AAC GGA GTC TAATCT CGA GC-3' (downstream, antisense; nts 9565-9584). Primer positions for ZYMV are relative to the full length sequence of the California strain (Balint, Plooy

& Steele, 1990; total 9593 nts, GenBank L31350) although this sequence was not used in the original primer design.

Immunocapture and RT-PCR

Microfuge tubes (Treff Lab) were coated with virus specific immunoglobulins, usually at 2-4 ng/ml, in carbonate buffer, pH 9.6, for either 3-4 hrs at 30°C or overnight at 4°C. After washing three times with PBS-Tween (PBS with 0.5 ml/l Tween 20) leaf samples, ground at a suitable dilution (usually 0.1 g to 5 ml) in PBS-Tween, were added and incubated as above. After again washing three times with PBS-Tween, tubes were washed once briefly with water and the RT/PCR reactants added (200 µM dNTPs, 1.5 mM magnesium chloride, 3 µl/ml Triton X-100, 1xPCR reaction buffer, 1 U Taq polymerase, 0.25 U AMV reverse transcriptase (both Gibco/BRL, Life Technologies, Scotland) 10 pmoles each primer and 2 µl PBS-Tween in a final volume of 25 µl). After overlaying with mineral oil, tubes and contents were heated according to the following: 42°C/45 min; 92°C/2 min; 58°C/1 min-72°C/2 min-92°C/1 min for 35 cycles; 58°C/1 min; 72°C/5 min.

PCR products were initially analysed by electrophoresis in an agarose gel (usually 15 g/l BioRad 162-0126), stained with ethidium bromide (0.5 µg/ml) and photographed in UV light. For RFLP analysis, aliquots (2 to 7 µl, depending on the concentration of product as judged subjectively from the initial analysis) of the PCR reactions (without purification or concentration) were incubated with appropriate restriction endonucleases and buffers according to the manufacturer's instructions, in final volumes of 10 µl, for 1 to 2 hrs; the products were then analysed by electrophoresis in an agarose gel (Metaphor, FMC BioProducts,15 to 20 g/l), stained and photographed as above.

RESULTS

With both pairs of primers, the RT-PCR product from the limited number of isolates tested were approximately of the sizes predicted (1210 and 1045 bp with primers 252/253 and 250/251 respectively) but the serologically distinct isolate from Reunion did not give a product (a further set of primers from elsewhere in the genome have given product from total leaf nucleic acids from plants infected with this isolate but not from immunocaptured particles; data not shown). Of the restriction endonucleases tested, *Hpa*II and *Spe*I gave the most easily distinguished RFLP patterns with the PCR products (using primers 252/253) from isolates Fa and Wk (Figure 1). When these enzymes were used to digest the products from artificial mixtures of immunocaptured particles of Fa and Wk the relative proportions could be quantitated visually from the resulting RFLP patterns (Figure 2).

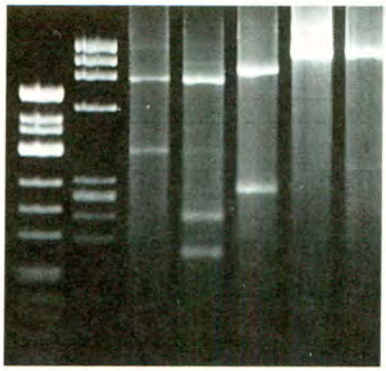


Figure 1. Typical RFLPs which distinguish ZYMV:Wk and ZYMV:Fa. Four microlitres of RT-PCR reaction was incubated with restriction endonucleases and appropriate buffers for 2 h at 37°C and then the DNA fragments separated by electrophoresis in a 20 g/l agarose (Metaphor) gel. Lanes: 1, DNA markers (ϕX174/*Hin*fI); 2, DNA markers (ϕX174/*Hae*III); 3, Wk/*Hpa*II; 4, Fa/*Hpa*II; 5, Wk/*Spe*I; 6, Fa/*Spe*I; 7, Wk/control.

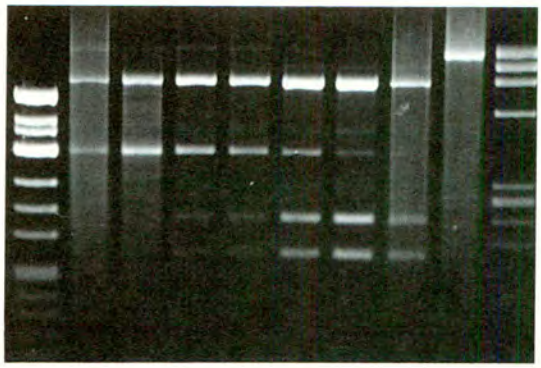


Figure 2. Differentiation of ZYMV:Wk and ZYMV:Fa immunocaptured from plant extracts mixed in known proportions. PCR product in all lanes except 9, control digested with *Hpa*II prior to electrophoresis. Lanes: 1, DNA markers (ϕX174/*Hin*fI); 2, Wk/100%; 3, Wk/90% - Fa/10%; 4, Wk/66% - Fa/33%; 5, Wk/50% - Fa/50%; 6, Wk/33% - Fa/66%; 7, Wk/10% - Fa/90%; 8, Fa/100%; 9, Fa/control; 10, DNA markers (ϕX174/*Hae*III).

DISCUSSION

The major objective was to distinguish and quantify ZYMV isolates in mixtures; typing unknown isolates was a low priority because in experimental cross-protection experiments both protecting and challenge isolates are selected and well-characterised. In this study, approximate quantification of the relative proportions of the two isolates was obtained by visual examination of photographs of the restriction endonuclease digests. This was simple and rapid; greater precision may be possible by use of image analysis techniques but these were not tried. The two main isolates studied here (Wk and Fa) were those already in experimental use and show that even with limited sequence data available (with none from these isolates) useful primers could be designed and that suitable RFLP differences could be readily identified.

Only limited sequence data was available at the time of primer design and this, combined with requirement to distinguish isolates, precluded the alternative approach of designing isolate specific primers giving products of discrete, identifiable lengths. The primers produced proved effective in immunocapture RT-PCR for for use with most isolates (the exception being the serologically distinct Reunion isolate of ZYMV, for which the immunotrapping may also be less efficient). Suitable RFLPs were readily identified, using only a limited range of restriction endonucleases, despite the isolates being serologically indistinguishable.

The immunocapture RT-PCR procedure described is rapid (it can be completed in less than two working days) and effective at distinguishing isolates and is probably widely applicable to strain identification. RFLP analysis of PCR products has been used to distinguish isolates which are well characterised (*e.g.* plum pox virus, Wetzel *et al.*, 1991) but our work clearly demonstrates that it is a useful approach even when sequence data is limited and not available for the isolates of interest.

In field trials the ability to distinguish the mild isolate from unselected challenge isolates would also be helpful. The method is currently being used to investigate field infections of ZYMV in the presence and absence of plants inoculated with ZYMV:Wk.

ACKNOWLEDGEMENTS

This work was funded by the Ministry of Agriculture, Fisheries and Food (MAFF). We are grateful to Dr T. Candresse for useful discussions and advice and thank Dr. H. Lecoq for providing ZYMV isolates. Non-indigenous ZYMV isolates are held under MAFF licence no. 1227C/1173/94.

REFERENCES

Balint, R; Plooy I; Steele, C 1990 The nucleotide sequence of zucchini yellow mosaic potyvirus. *Abstract P84-017- VIII International Congress of Virology.*

Jansen, R W; Siegl, G; Lemon, S M 1990 Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proceedings of the National Academy of Sciences, USA* **87**: 2867-2871.

Lecoq, H; Lemaire, J M; Wipf-Scheibel, C 1991 Control of zucchini yellow mosaic virus in squash by cross protection. *Plant Disease* **75**: 208-211.

Schrijnwerkers, C C F M; Huijberts, N; Bos, L 1991 Zucchini yellow mosaic virus; two outbreaks in the Netherlands and seed transmissibility. *Netherlands Journal of Plant Pathology* **97**: 187-191.

Walkey, D G A; Lecoq, H; Collier, R; Dobson, S 1992 Studies on the control of zucchini yellow mosaic virus in courgettes by mild strain protection. *Plant Pathology* **41**: 762-771

Wetzel, T; Candresse, T; Ravelonandro, M; Dunez, J 1992 A polymerase chain reaction assay adapted to plum pox potyvirus detection. *Journal of Virological Methods* **33**: 355-365.

Wetzel, T; Candresse, T; Macquaire, G; Ravelonandro, M; Dunez, J 1991 A highly sensitive polymerase chain reaction method for plum pox potyvirus detection. *Journal of Virological Methods* **39**: 27-37.

EXPLOITATION OF MOLECULAR DIAGNOSTICS TO DISCRIMINATE BETWEEN BEET MILD YELLOWING AND BEET WESTERN YELLOWS LUTEOVIRUSES.

M STEVENS, H G SMITH
IACR-Broom's Barn, Higham, Bury St. Edmunds, Suffolk, IP28 6NP, UK

O LEMAIRE, E HERRBACH
INRA Colmar, Station Grandes Cultures, 28 rue de Herrlisheim, 68021 Colmar, France

ABSTRACT

Total RNA was extracted from six BMYV and four BWYV isolates , and reverse transcribed using primers designed either to the coat protein gene (ORF4) or the first open reading frame (ORF1). A product of 563 base pairs was observed for all BMYV and BWYV isolates when the cDNA of the coat protein gene was amplified by the polymerase chain reaction (RT-PCR). The BMYV ORF1 primers amplified five of the six BMYV isolates, but the BWYV ORF1 primers did not amplify any BMYV DNA fragments. However, only one of the four BWYV isolates was amplified when the BWYV ORF1 primers were used. Restriction fragment length polymorphism analysis of the coat protein products revealed that the enzyme *Sma1* cut the four BWYV isolates as well as the BMYV isolate not amplified by the BMYV ORF1 primers, but not any of the other BMYV isolates. Conversely, restriction enzyme *Mse 1* cut only the coat protein PCR products of isolates not cut by *Sma 1*. These results show that there is sequence variability within ORF1 and ORF4 for both BMYV and BWYV isolates.

INTRODUCTION

Beet mild yellowing luteovirus (BMYV) is an important disease of sugar beet, which can decrease the yields of infected plants by up to 30%. The virus is transmitted by aphids, principally the peach potato aphid (*Myzus persicae*), although other aphid species do play a role in the epidemiology of the disease, for example the potato aphid (*Macrosiphum euphorbiae*). BMYV is very closely related to beet western yellows luteovirus (BWYV), which is transmitted by the same aphid species, and BWYV is thought to be the main cause of yellowing of sugar beet in the western states of the USA. The majority of isolates of BWYV which have been identified in Europe do not infect sugar beet, but are commonly found in brassica crops such as oilseed rape (Smith & Hinckes, 1985). Serologically BMYV and BWYV cannot be distinguished using polyclonal antiserum, and attempts to develop a specific monoclonal antibody using either virus as the antigen, have resulted in antibodies that recognise common beet luteovirus epitopes (Smith *et al.*, 1996). A monoclonal antibody produced in response to barley yellow dwarf luteovirus (BYDV-PAV-IL-1) was found to distinguish BMYV from BWYV (D'Arcy *et al.*, 1989; Smith *et al.*, 1991), although more recent studies have shown that it does not detect all strains of BMYV (Stevens *et al.*, 1994).

Recent molecular studies by Guilley *et al.*, (1995) have revealed sufficient sequence heterology between BMYV and BWYV, particularly at the 5' end of the genomes, to indicate that they are two distinct viruses, rather than BMYV being a pathotype of BWYV as suggested by Casper

(1988). Previously, this heterology was exploited by Herrbach *et al.* (1991) and Lemaire *et al.* (1995) for the development of specific BMYV and BWYV RNA probes within ORF1, although, as with monoclonal antibody BYDV-PAV-IL-1, the BMYV-specific probe did not hybridise to the same extent with all BMYV isolates. The need for specific and sensitive molecular diagnostic methods to distinguish between BMYV and BWYV in plants and aphids has resulted in further development of the reverse-transcription polymerase chain reaction (RT-PCR), first described for BMYV and BWYV by Jones *et al.* (1991). This paper describes the use of new RT-PCR primers, designed to the coat protein gene (ORF4) and the first open reading frame (ORF1), and the subsequent analysis of the PCR products, using restriction fragment length polymorphisms (RFLP's) to demonstrate inter- and intra-specific variation amongst BMYV and BWYV.

MATERIALS AND METHODS

Virus isolates

Yellow leaves were collected randomly from six sugar-beet plants in a commercial crop grown at Broom's Barn in 1995; these were confirmed to contain BMYV by TAS-ELISA as described by Smith *et al.* (1996). Four isolates of BWYV, collected from autumn-sown oilseed rape crops and maintained in glasshouse-grown oilseed rape, were also used. Glasshouse-grown oilseed rape and sugar beet, not exposed to aphids were used as negative controls.

Isolation of total RNA

Two methods were compared for the extraction of total RNA from infected and healthy plant material. The first (Method 1) was based on that developed by Robertson *et al.* (1991). Leaf material (0.125g) was ground to a fine powder in liquid nitrogen and 1 ml of grinding buffer (0.1M NaCl, 0.1M glycine, 10mM EDTA, pH 9.5) was added. To 0.7 ml of the homogenate an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. Following centrifugation, 333 µl of the aqueous phase were mixed with 33 µl 3M sodium acetate and 1ml of ice-cold 100% ethanol, the tubes were then placed at -70°C for at least one hour. The precipitate was collected by centrifugation, washed once with 70 % ethanol and resuspended in 20 µl of double distilled water. The second method (Method 2) followed the instructions provided by Qiagen, Surrey, England for their RNeasy plant total RNA extraction kit.

Primers

The coat protein primers (ORF4+ and ORF4$^-$) were designed around the start and stop codons of the conserved coat protein sequences of BWYV-FL1 (EMBL access number X13063, Veidt *et al.*, 1988) and BMYV-2ITB (EMBL access number X83110, Guilley *et al.*, 1995). These primers flank the PCR fragment of 563 base pairs. Two further sets of primers were developed around the start and stop codons of BWYV-FL1 and BMYV-2ITB ORF1 (BWORF1+ and $^-$ and BMORF1+ and $^-$ respectively). According to the literature, these primers produce RT-PCR fragments of 750 and 720 base pairs respectively.

cDNA synthesis and PCR reaction

Two microlitres of total plant and viral RNA, from both extraction methods, were diluted to 12.5 µl in double distilled water containing 10 pmols of 3' ($^-$) primer. A total of 7.5 µl of reverse

transcription mix (1 µl water, 4 µl 5*RT buffer (Promega, England), 2 µl 0.1M DTT, 1 µl 10 mM dNTP's, 0.5 µl (100 Units) reverse transcriptase and 0.5 µl (20 Units) Rnasin (Promega, England)) was added and the mixture was incubated for 1 hour; 30 µl of distilled water were added to the synthesised cDNA.

cDNA (2.5 µl) was added to the PCR reaction mixture (37.75 µl distilled water, 8.0 µl 10*PCR buffer containing 25 mM $MgCl_2$ (Boehringer-Mannheim, Germany), 1.0 µl dNTP's, 0.25 µl (200mM) 5' (+) primer, 0.25 µl (200mM) 3' (-) primer and 0.2 U Taq polymerase (Boehringer-Mannheim, Germany)). The reaction mixture was covered with 25 µl of mineral oil and placed into a Hybaid Thermal Cycler.The standard thermal cycle consisted of: 1 cycle for 5 minutes at 94°C; 30 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, and 1 cycle at 72 °C for 10 minutes. The PCR products were separated by electrophoresis in 1% agarose gels in 1*TBE and stained with ethidium bromide.

RFLP analysis of coat protein PCR products

The amplified coat protein fragments were digested with *Taq 1*, *Sma 1* and *Mse 1* according to the instructions provided by Boehringer-Mannheim. These enzymes were selected by using the GCG package (University of Wisconsin) as enzymes which would digest BMYV and BWYV, BWYV only, or BMYV only respectively. Digests were visualised by electrophoresis on 3% Nusieve 3:1 agarose gels (Flowgen, England) stained with ethidium bromide and run in 1*TBE buffer.

RESULTS

Amplification of the BMYV and BWYV coat protein gene (ORF 4)

The two total RNA extraction procedures used were both equally successful at isolating viral RNA, indicated by the observation of a PCR product of 563 base pairs for all BMYV and BWYV isolates tested (Table 1); no other bands were observed for these samples. No products were seen with either healthy sugar-beet or oilseed rape preparations or water controls.

Amplification of the BMYV and BWYV ORF 1

An RT-PCR product was observed for five out of six BMYV isolates when the BMYV ORF1 primers were used (Table 1); no products were observed when the BWYV ORF1 primers were tested. However, only one of the four BWYV isolates gave a product when amplified with the BWYV ORF1 primers (Table 1). No products were produced from the BWYV isolates with the BMYV ORF1 primers. No products were observed with either healthy sugar beet, oilseed rape or water controls.

RFLP analysis of the coat protein PCR product

When digested with the enzyme *Taq 1*, the RFLP's of the coat protein products of BMYV and BWYV were identical. Enzyme *Mse 1* digested all BMYV isolates except the isolate that was not amplified by the BMYV ORF1 primers (Table 1), whereas *Mse 1* did not digest any of the BWYV coat protein PCR products. Conversely, restriction enzyme *Sma 1* cut all BWYV isolates and the BMYV isolate that was not cut by *Mse 1*. *Sma 1* did not cut the other BMYV

isolates (Table 1).

Table 1 Summary of the RT-PCR and RFLP results for the six BMYV and four BWYV isolates.

Isolates	PCR amplification with primers			Digestion of ORF4 by		
	ORF4	BMYVORF1	BWYVORF1	*Taq I*	*Sma I*	*Mse I*
BMYV-1	+	+	-	+	-	+
BMYV-2	+	+	-	+	-	+
BMYV-3	+	+	-	+	-	+
BMYV-4	+	+	-	+	-	+
BMYV-5	+	+	-	+	-	+
BMYV-6	+	-	-	+	+	-
BWYV-1	+	-	+	+	+	-
BWYV-2	+	-	-	+	+	-
BWYV-3	+	-	-	+	+	-
BWYV-4	+	-	-	+	+	-

+ = successful PCR amplification or digestion by enzyme
- = no amplification or digestion

DISCUSSION

Beet mild yellowing virus and beet western yellows virus are closely related luteoviruses which differ in their host range of commercial crop plants and weeds, and exist as a number of distinct strains throughout the world. Therefore, it is important to be able to characterise and distinguish BMYV and BWYV strains for epidemiological and breeding studies, and to detect the viruses in mixed infections, and in aphid vectors which may be carrying more than one isolate. A method was developed for distinguishing between BMYV and BWYV, based on restriction analysis of the BMYV/BWYV coat protein gene, amplified by reverse transcription and the subsequent polymerase chain reaction. The method successfully amplified the whole coat protein gene of all isolates tested, and digestion of these products revealed restriction enzymes that gave unique patterns for different isolates of BMYV and BWYV.

The primers designed around the start and stop codons of BMYV ORF1 also appeared to be BMYV specific as all attempts to amplify BWYV with these primers were unsuccessful. However, these primers did not amplify the BMYV isolate that had the same restriction profiles as the BWYV isolates within the coat protein gene. This suggests that there are further differences between the two BMYV strains at the 5' end of their genomes. The gene encoded by ORF1 is thought to be either the host range determinant or linked to symptomatology (Mayo *et al.*, 1989; Van der Wilk *et al.*, 1989), and studies are in progress to compare the two BMYV sequences. Similarly, the BWYV ORF 1 primers only amplified one of the four BWYV isolates. These

primers were designed from the BWYV-FL1 sequence (Veidt *et al.*, 1988) which shows that there is more sequence heterology among these isolates of BWYV than between those of BMYV at the 5' end of their genomes.

ACKNOWLEDGEMENTS

The first author was the recipient of a BBSRC/INRA fellowship enabling collaboration between IACR-Broom's Barn and INRA Colmar. The work was funded from the UK Home Grown Sugar Beet Research and Education Fund. IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

REFERENCES

Casper, R (1988) Luteoviruses. In: *The Plant Viruses, Polyhedral Virions with Monopartite RNA genomes*, R Koenig (ed), New York : Plenum Press, pp. 235-258.

D'Arcy, C J; Torrance, L; Martin, R R (1989) Discrimination among luteoviruses and their strains by monoclonal antibodies and identification of common epitopes. *Phytopathology*. **79**, 869-873.

Guilley, H; Richards, K E; Jonard, G (1995) Nucleotide sequence of beet mild yellowing virus RNA. *Archives of Virology*. **140**, 1109-1118.

Herrbach, E; Lemaire, O; Ziegler-Graff, V; Lot, H; Rabenstein, F; Bouchery, Y (1991) Detection of BMYV and BWYV using monoclonal antibodies and radioactive RNA probes, and relationships among luteoviruses. *Annals of Applied Biology*. **118**, 127-138.

Jones, T D; Buck, K W; Plumb, R T (1991) The detection of beet western yellows virus and beet mild yellowing virus in crop plants using the polymerase chain reaction. *Journal of Virological Methods*. **35**, 287-296.

Lemaire, O; Herrbach, E; Stevens, M; Bouchery Y; Smith H G (1995) Detection of sugar-beet infecting beet mild yellowing luteovirus isolates with a specific RNA probe. *Phytopathology*. **85**, 1513-1518.

Mayo, M A; Robinson, D J; Jolly, C A; Hyman, L (1989) Nucleotide sequence of potato leafroll luteovirus RNA. *Journal of General Virology*. **70**, 1037-1051.

Robertson, N L; French, R; Gray, S M (1991) Use of group-specific primers and the polymerase chain reaction for the detection and identification of luteoviruses. *Journal of General Virology*. **72**, 1473-1477.

Smith, H G; Barker, I; Brewer, G; Stevens, M; Hallsworth, P B (1996) Production and evaluation of monoclonal antibodies for the detection of beet mild yellowing luteovirus and related strains. *European Journal of Plant Pathology*. **102**, 163-169.

Smith, H G; Hinckes, J A (1985) Studies on beet western yellows virus of oilseed rape (*Brassica*

napus ssp.*oleifera*) and sugar beet (*Beta vulgaris*). *Annals of Applied Biology*. **107**, 473-484.

Smith, H G; Stevens, M; Hallsworth, P B (1991) The use of monoclonal antibodies to detect beet mild yellowing virus and beet western yellows virus in aphids. *Annals of Applied Biology*. **119**, 295-302.

Stevens, M; Smith, H G; Hallsworth, P B (1994) Identification of a second distinct strain of beet mild yelllowing luteovirus using monoclonal antibodies and transmission studies. *Annals of Applied Biology*. **125**, 515-520.

Van der Wilk , F; Huisman, M J; Cornelissen, B J C; Huttinga, H; Goldbach, R (1989) Nucleotide sequence and organisation of potato leafroll virus genomic RNA. *FEBS Letters*. **245**, 51-56.

Veidt, I; Lot, H; Leiser, R M; Scheidecker, D; Guilley, H; Richards, K; Jonard, G (1988) Nucleotide sequence of beet western yellows virus RNA. *Nucleic Acids Research*. **16**, 9917-9932.

DNA PROFILING FOR VARIETAL IDENTIFICATION IN CROP PLANTS.

D LEE, J C REEVES, R J COOKE
NIAB, Huntingdon Road, Cambridge CB3 0LE

ABSTRACT

Two approaches to DNA profiling for identification of and discrimination between varieties of oilseed rape and barley have been evaluated and compared. The analysis of RFLPs (with various types of probe) and RAPDs (with a range of primers) will readily distinguish between varieties of these two species at high levels of separation. The potential of such methods, especially in the context of the statutory distinctness testing of varieties, is discussed.

INTRODUCTION

Before they can be marketed within the EU, newly bred varieties (cultivars) of crop plants must undergo statutory testing to determine their eligibility for inclusion on the National List of Varieties. Part of this testing requires that varieties are not only shown to be distinct (D) from others known to exist, but also that they demonstrate uniformity (U) and stability (S) in the characteristics used to distinguish and describe them.

Currently, DUS testing involves the comparison of new (candidate) and existing varieties by the recording of a number of phenotypic characters or descriptors in tests in which candidate and existing varieties are grown side by side. This can be a time-consuming and expensive process, requiring large areas of land and highly skilled personnel making what are often subjective decisions. Many of the characters used are multi-genic, quantitative or continuous and their expression can be altered by environmental factors, which necessitates replication of observation. Further, in some species the number of characters is limited or is no longer sufficient for discrimination of all varieties. There are thus compelling reasons to find more rapid and cost-effective procedures to augment this approach (Cooke, 1995).

The objective of this research was to evaluate and compare methods for assessing DNA polymorphism in crop species in a structured manner. Oilseed rape and barley were used as model systems and the research concentrated on two approaches: (a) probe-based technologies, i.e. restriction fragment length polymorphisms (RFLP), using various sources of probes, and (b) amplification-based technologies, i.e. the use of random amplified polymorphic DNA (RAPD). The techniques were examined for their potential both for variety identification/ characterisation and for incorporation into DUS testing schemes.

MATERIALS AND METHODS

Plant material. Seed of oilseed and forage rape (*Brassica napus*) and barley *(Hordeum vulgare*) varieties was obtained from the reference collections held at NIAB. Seedlings were grown in a greenhouse until the fourth (rape) or first (barley) true leaf stage.

DNA Extraction. DNA was extracted from 50-100 seedlings of 62 varieties of rape (representing much of the UK National List) and 36 varieties of barley, by grinding the seedlings in liquid nitrogen and performing chloroform/phenol extractions.

RFLP analysis. DNA was digested and then fractionated by electrophoresis using 1% agarose gels in 40 mM Tris: 5 mM Na acetate buffer, pH 7.9, containing 1 mM EDTA, at 2 V/cm for 16 hours. The DNA fragments were Southern blotted onto nylon membranes and fixed by UV cross-linking. DNA probes were labelled by the random oligo-labelling method (Feinberg and Vogelstein, 1984). Hybridisations were carried out essentially as described by Ellis *et al.* (1988).

RAPD analysis. RAPD analysis was performed using standardised conditions as given in Lee *et al.* (1996). All amplifications were performed in duplicate.

Gel evaluation/data analysis. Data evaluation was carried out either visually or using the software 'GelCompar' (Applied Maths, Kortrijk, Belgium) (Lee *et al.*, 1996). Data matrices were constructed, defining all of the bands present in the varieties analysed and classifying each variety in terms of the bands present or absent. The discrimination potentials of the various approaches were calculated on the basis of separation coefficients, defined as s = number of pairs of varieties separated/total number of pairs. Similarity coefficients were calculated using the Jaccard coefficient and cluster analysis was performed using the UPGMA method.

RESULTS.

RFLP in oilseed rape.

Of the genomic clones used as probes, two (pN180 and pN216) were found to be especially useful for discriminating between varieties of oilseed and forage rape. Probe pN180 produced 22 different bands from the 62 varieties analysed, with six bands common to all varieties. Each variety produced 11-15 bands. The 62 varieties produced 46 different patterns, of which 39 were unique. Probe pN216 produced 16 different bands, with three monomorphic bands. Within the 62 varieties tested, 26 recognisably different banding patterns could be detected, with 13 varieties in the largest group and with 16 varieties having a unique banding pattern. One variety possessed a unique DNA band. The separation coefficients of these two probes are shown in Table 1.

Table 1. Separation coefficients (s) for RFLP analysis of 62 oilseed rape varieties using two genomic clones as probes following digestion with *HindIII*.

Probe	s(%)
A - pN180	96.6
B- pN216	93.4
C - A+B combined	99.2

Cluster analysis based on the combined data from these two probes separated the 62 varieties into two main groups, corresponding to winter and spring types, with the forage rapes not clustering predominantly with either group. This separation into winter and spring varieties may indicate that RFLP analysis using these two probes, and involving 38 alleles (if it is assumed that a band can be equated to an allele), reflects the genetic relationship between varieties. This is supported by the fact that the clustering brought together varieties that are known to be close in parentage or arise from the same breeding programme.

The use of these two probes uniquely separated all of the varieties apart from one group of four varieties and seven further pairs. These could all be distinguished either by using a different restriction enzyme in combination with the two probes, or by the use of other probes. Perhaps of particular interest were the results obtained with simple oligonucleotide repeats. For instance, probing with $(GATA)_5$ enabled clear discrimination between all but one pair of the otherwise identical varieties.

RAPD in oilseed rape.

Initial results on the use of RAPD to distinguish between oilseed rape varieties and the application of the 'GelCompar' gel documentation system have been reported elsewhere (Lee *et al.*, 1996). The following primers were selected for use, after an initial screening of 18 primers: primer 6 (ACGTAGCGTC), primer 18 (GCGCGGTACT), primer 33 (CGGTAGCCGC), primer 62 (ATCTTCCGCC), primer 70 (CGTAGTGGTG). Although care is needed to standardise experimental conditions, especially choice of polymerase enzyme and quality of DNA, RAPDs banding patterns were found to be repeatable, particularly within a restricted region (usually between 500 bp to 2 kb). Furthermore, the profiles showed clear differences between varieties, indicating potential utility for variety identification. Table 2 shows the separation coefficients for the five primers above, following analysis using 'GelCompar'. These were obtained using a particular set of band acceptance criteria, i.e. that before it is included in the analysis, a band must be 10% above the background level of the gel in intensity (peak height following scanning) and must also exceed 1% of the total area under the scan. Varying the acceptance criteria gives different separation rates (Lee *et al.*, 1996).

Table 2. Separation coefficients for RAPD analysis of 50 oilseed rape varieties using five primers.

Primer	*s*(%)
6	99.5
18	97.4
33	99.5
62	99.8
70	99.8

Uniformity of profiles.

Any character used to demonstrate distinctness in a DUS test must also be shown to be 'sufficiently uniform', taken on a plant by plant basis. To investigate the degree of uniformity of RAPD profiles in oilseed rape varieties, DNA isolated from single plants within certain varieties was analysed using the primers above. The results showed clearly that there was variation within some varieties for their RAPD profiles when using certain primers (Lee *et al.*, 1996). This lack of uniformity may cause a problem in the context of DUS testing and is considered further below.

RFLP in barley

A collection of mostly single copy barley probes, derived from different linkage groups, was evaluated against 36 barley varieties. The separation coefficients for three of these probes, alone and in combination, are shown in Table 3.

Table 3. Separation coefficients for RFLP analysis of 36 barley varieties using three single copy probes.

Probe	*s*(%)
A	63
B	53
C	65
A+B+C	91

RAPD in barley

RAPD analysis of barley varieties revealed relatively little polymorphism in comparison to oilseed rape. Improved band separations and enhanced polymorphism were achieved by pre-digestion of DNA prior to amplification. Even so, only moderate separation coefficients between 36 varieties were obtained (see Table 4), although these were improved by combining data from more than one primer.

Table 4. Separation coefficients for RAPD analysis of 36 barley varieties using three different primers.

Primer	*s*(%)
A-UCD 60	24
B-ATC 43	67
C-G19	64
A+B+C	95

DISCUSSION

This research has clearly demonstrated that both RFLP and RAPD possess considerable potential for discrimination, particularly for varieties of oilseed rape, and thus their uses within the DUS testing context need to be carefully evaluated. Notwithstanding doubts about the reproducibility of RAPD and difficulties in the automated and objective evaluation of gels (see Lee *et al.*, 1996), it is apparent that both techniques are highly discriminating, convenient, relatively rapid, suitable for side by side comparisons of samples and can be carried out at any time of the year. These represent considerable advantages over most of the morphological characters currently used in DUS tests and a morphological character with such attractive features would surely be treated very seriously as a potential DUS tool.

It is recognised that there are reservations in some quarters about the use of profiling techniques for variety registration, which seem to revolve around perceived problems connected with insufficient coverage of the genome and lack of knowledge of the genetic control of the marker(s). However, it is doubtful that any of the currently used morphological characters fulfil such criteria either and so, judged against the same standards as other DUS characters, molecular markers must still be seen as being potentially advantageous and worthy of further study.

Our work to date has clearly demonstrated that varieties can be heterogeneous in their DNA profiles as determined using RAPD. Whilst this is not entirely unexpected, it does raise potential difficulties for DUS testing, if the criteria presently used for assessment of uniformity were maintained. The genetic structure of varieties of many crops means that there is an inherent variability within them, such that they will not be completely homogeneous in discontinuous characters such as a DNA profile when examined on a plant by plant basis. There are a number of ways of approaching this problem:

(i) it could be decided that this lack of uniformity precludes the use of profiling techniques; (ii) it could be accepted that the level of non-uniformity exhibited by currently registered varieties (which would need to be determined empirically) represented a baseline, which candidate varieties in the future would not be allowed to exceed; (iii) it could be suggested that from a certain date, all future candidates would have to be uniform for the particular profiling character; (iv) it could be suggested that from a certain date, those candidates for which the profiling data was the sole distinctness criterion would have to be uniform for that profiling character; (v) it could be accepted that the repeatability (i.e. stability) of the differences between varieties is more important than plant to plant uniformity. Thus if the variability within a variety were stable from generation to generation, then this could be accepted as evidence of sufficient uniformity within that variety. This would be recognising that the examination of uniformity is at least partly to ensure that the distinguishing features of a variety are maintained during multiplication and commercialisation. Hence it is stability rather than uniformity *per se* which is essential.

This last point would represent a change in the philosophy underlying aspects of DUS testing, but might be biologically, as well as practically, desirable. The insistence on complete uniformity of DNA profile within varieties, in addition to being in all probability

difficult to achieve, is also of doubtful biological and agronomic value. Profiling techniques are ideally suited to the rapid assessment of stability, since different generations can be screened and compared side by side on the same gel.

Even if these problems eventually preclude the use, for instance, of RAPD for registration, there could still be important applications of the techniques in verification of identity and checking stability. The databases and clusters produced by profiling may also be utilised in areas that are somewhat peripheral to DUS testing itself, but are nonetheless essential in their own right, such as the grouping of existing and candidate varieties and selection of appropriate controls. Some of the problems may be diminished by the use of the 'second generation' profiling techniques such as sequence tagged site microsatellites, which may be more cost-effective, amenable to automation and easier to score reliably. We are currently investigating these methods in oilseed rape.

In conclusion, the use of DNA markers as a diagnostic tool for variety identification and DUS testing is feasible and more research is needed to investigate (a) other profiling methods, (b) automated methods of gel evaluation, recording, etc., (c) applications of databases of DNA profiles within the DUS testing context, (d) appropriate means to utilise DNA markers within the variety registration system.

ACKNOWLEDGEMENTS

Dr Derek Lydiate and Dr Noel Ellis (JIC, Norwich) and the Federal Centre for Breeding Research on Cultivated Plants (BAZ, Grunbach, Germany), are thanked for provision of probes. This work was supported by a grant from the Ministry of Agriculture, Fisheries and Food, under project VS0105.

REFERENCES

Cooke, R J (1995). Varietal identification of crop plants. In: *New Diagnostics in Crop Sciences,* J H Skerritt, and R Appels (eds), Wallingford: CAB International, pp. 33-63.

Ellis, T H N; Lee, D; Thomas, C M; Simpson, P R; Cleary, W G; Newman, M-A; Burcham, K W G (1988). 5S rRNA genes in *Pisum*: Sequence, long range and chromosomal organisation. *Molecular and General Genetics.* **214**, 333-342.

Feinberg, A P; Vogelstein, B (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry.* **132**, 6-13.

Lee, D; Reeves, J C; Cooke, R J (1996) DNA profiling and plant variety registration: 1. The use of random amplified DNA polymorphisms to discriminate between varieties of oilseed rape. *Electrophoresis* (in press).